

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
17 July 2003 (17.07.2003)

PCT

(10) International Publication Number  
WO 03/057914 A2

- (51) International Patent Classification<sup>7</sup>: C12Q 1/68
- (21) International Application Number: PCT/GB03/00034
- (22) International Filing Date: 7 January 2003 (07.01.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
0200239.2 7 January 2002 (07.01.2002) GB  
0214124.0 19 June 2002 (19.06.2002) GB
- (71) Applicant (*for all designated States except US*):  
NORCHIP A/S [NO/NO]; Industriveien 8, N-3490  
Klokkarstua (NO).
- (71) Applicant (*for MG only*): ALLARD, Susan, Joyce  
[GB/GB]; Boulton Wade Tennant, Verulam Gardens, 70  
Gray's Inn Road, London WC1X 8BT (GB).
- (72) Inventor; and
- (75) Inventor/Applicant (*for US only*): KARLSEN, Frank  
[NO/NO]; Hovkastet 2, N-3490 Klokkarstua (NO).
- (74) Agents: BALDOCK, Sharon, Claire et al.; Boulton Wade  
Tennant, Verulam Gardens, 70 Gray's Inn Road, London  
WC1X 8BT (GB).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC,  
VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI,  
SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN,  
GQ, GW, ML, MR, NE, SN, TD, TG).
- Declaration under Rule 4.17:**  
— *of inventorship (Rule 4.17(iv)) for US only*
- Published:**  
— *without international search report and to be republished  
upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/057914 A2

(54) Title: METHOD FOR DETECTING HUMAN PAPILLOMARVIRUS MRNA

(57) Abstract: An in vitro method is provided for screening human female subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts from the E6 and optionally the L1 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or E6 mRNA are scored as being at risk of developing cervical carcinoma. Kits for carrying out such methods are also provided.

- 1 -

METHOD FOR DETECTING HUMAN PAPILLOMAVIRUS mRNAField of the invention

5 The present invention relates to *in vitro* methods of screening human subjects in order to assess their risk of developing cervical carcinoma.

Background to the invention

10 Cervical carcinoma is one of the most common malignant diseases world-wide and is one of the leading causes of morbidity and mortality among women (Parkin DM, Pisani P, Ferlay J (1993) Int J Cancer 54: 594-606; Pisani P, Parkin DM, Ferlay J (1993) Int J Cancer 55: 891-903). 15,700 new cases of invasive  
15 cervical cancer were predicted in the United States in 1996, and the annual world-wide incidence is estimated to be 450,000 by the World Health Organization (1990). The annual incidence rate differs in different parts of the world, ranging from 7.6 per 100,000 in western  
20 Asia to 46.8 per 100,000 in southern Africa (Parkin et al., 1993 *ibid*).

The current conception of cervical carcinoma is that it is a multistage disease, often developing over  
25 a period of 10-25 years. Invasive squamous-cell carcinoma of the cervix is represented by penetration through the basal lamina and invading the stroma or epithelial lamina propria. The clinical course of cervical carcinoma shows considerable variation.  
30 Prognosis has been related to clinical stage, lymph node involvement, primary tumour mass, histology type, depth of invasion and lymphatic permeation (Delgado G, et al., (1990) Gynecol Oncol 38: 352-357). Some patients with less favourable tumour characteristics  
35 have a relatively good outcome, while others suffer a fatal outcome of an initially limited disease. This

- 2 -

shows a clear need for additional markers to further characterise newly diagnosed cervical carcinomas, in order to administer risk-adapted therapy (Ikenberg H, et al., Int. J. Cancer 59:322-6. 1994).

5

The epidemiology of cervical cancer has shown strong association with religious, marital and sexual patterns. Almost 100 case-control studies have examined the relationship between HPV and cervical neoplasia and almost all have found positive associations (IARC monographs, 1995). The association is strong, consistent and specific to a limited number of viral types (Munoz N, Bosch FX (1992) HPV and cervical neoplasia: review of case-control and cohort studies. IARC Sci Publ 251-261). Among the most informative studies, strong associations with HPV 16 DNA have been observed with remarkable consistency for invasive cancer and high-grade CIN lesions, ruling out the possibility that this association can be explained by chance, bias or confounding (IARC monographs, 1995). Indirect evidence suggested that HPV DNA detected in cancer cells is a good marker for the role of HPV infection earlier in the carcinogenesis. Dose-response relationship has been reported between increasing viral load and risk of cervical carcinoma (Munoz and Bosch, 1992 *ibid*). In some larger series up to 100% of the tumours were positive for HPV but the existence of virus-negative cervical carcinomas is still debatable (Meijer CJ, et al., (1992) Detection of human papillomavirus in cervical scrapes by the polymerase chain reaction in relation to cytology: possible implications for cervical cancer screening. IARC Sci Publ 271-281; Das BC, et al., (1993) Cancer 72: 147-153).

35

The most frequent HPV types found in squamous-cell cervical carcinomas are HPV 16 (41%-86%)

- 3 -

and 18 (2%-22%). In addition HPV 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 61, 66 and 68 are also found (IARC, monographs, 1995). In the HPV2000 International conference in Barcelona HPV 16, 18, 31 and 45 were  
5 defined as high risk, while HPV 33, 35, 39, 51, 52, 56, 58, 59, 68 were defined as intermediate risk (Keerti V. Shah. P71). The 13 high risk plus intermediate risk HPVs are together often referred to as cancer-associated HPV types.

10

A number of studies have explored the potential role of HPV testing in cervical screening (see Cuzick et al. A systematic review of the role of human papillomavirus testing withing a cervical screening  
15 programme. Health Technol Assess 3:14. 1999).

Reid et al., (Reid R, et al., (1991) Am J Obstet Gynecol 164: 1461-1469) where the first to demonstrate a role for HPV testing in a screening context. This  
20 study was carried out on high-risk women from sexually transmitted disease clinics and specialist gynaecologists, and used a sensitive (low stringency) Southern blot hybridisation for HPV detection. A total of 1012 women were enrolled, and cervicography  
25 was also considered as a possible adjunct to cytology. Twenty-three CIN II/III lesions were found altogether, but only 12 were detected by cytology (sensitivity 52%, specificity 92%). HPV testing found 16 high-grade lesions.

30

Bauer et al. (Bauer HM, et al., (1991) JAMA 265: 472-477) report an early PCR-based study using MY09/11 primers (Manos M, et al., (1990) Lancet 335: 734) in  
young women attending for routine smears (college  
35 students). They found a positive rate of 46% in 467 women, which was much higher than for dot blot assay (11%).

- 4 -

In a study using PCR with GP5/6 primers (Van Den Brule AJ, et al., (1990) J Clin Microbiol 28: 2739-2743) van der Brule et al. (Van Den Brule AJ, et al., (1991) Int J Cancer 48: 404-408) showed a very strong correlation of HPV positivity with cervical neoplasia as assessed by cytology. In older women (aged 35-55 years) with negative cytology the HPV positivity rate was only 3.5%, and this was reduced to 1.5% if only types 16, 18, 31 and 33 were considered, while women with histological carcinoma in situ were all HPV-positive, and 90% had one of the four above types. Women with less severe cytological abnormalities had lower HPV positivity rates in a graded way, showing a clear trend.

Roda Housman et al. (Roda Housman AM, et al., (1994) Int J Cancer 56: 802-806) expanded these observations by looking at a further 1373 women with abnormal smears. This study also confirmed increasing positivity rate with increasing severity of smear results. They also noted that the level of HPV heterogeneity decreased from 22 types for low-grade smears to ten "high-risk" types for high grade smears. This paper did not include any cytologically negative women, nor was cytological disease confirmed histologically.

Cuzick et al. (Cuzick J, et al., (1992) Lancet 340: 112-113; Cuzick J, et al., (1994) Br J Cancer 69: 167-171) were the first to report that HPV testing provided useful information for the triage of cytological abnormalities detected during random screening. In a study of 133 women, referral for colposcopy they found a positive predictive value of 42%, which was similar to that for moderate dyskaryosis. The results were most striking for HPV 16, where 39 of 42 HPV 16 positive women were found to

- 5 -

have high-grade CIN on biopsy. This study pointed out the importance of assessing viral load and only considered high levels of high-risk types as positive.

5           Cox et al. (Cox JT, et al., (1995) Am J Obstet  
Gynecol 172: 946-954) demonstrated a role for HPV  
testing using the Hybrid Capture™ system (DIGENE  
Corporation, Gaithersburg, MD, USA) for triaging women  
with borderline smears. This test was performed on  
10   217 such women from a college referral service, and a  
sensitivity of 93% was found for CINII/III compared  
with 73% for repeat cytology. High viral load was  
found to further improve performance by reducing false  
positives. When 5 RLU was taken as a cut-off, a PPV  
15   of approximately 24% was found with no loss of  
sensitivity.

          Cuzick et al. (Cuzick J, et al., (1995) Lancet  
345: 1533-1536) evaluated HPV testing in a primary  
20   screening context in 1985 women attending for routine  
screening at a family planning clinic. Sensitivity  
using type-specific PCR for the four common HPV types  
(75%) exceeded that of cytology (46%), and the PPV for  
a positive HPV test (42%) was similar to that for  
25   moderate dyskaryosis (43%).

          WO 91/08312 describes methods for determining the  
prognosis of individuals infected with HPV which  
comprise measuring the level of HPV activity by  
30   detecting transcripts of all or a portion of the E6  
and/or E7 HPV genes in a sample and comparing the  
measurements of HPV activity with a previously  
established relationship between activity and risk of  
progression to serious cervical dysplasia or  
35   carcinoma.

          WO 99/29890 describes methods for the assessment

- 6 -

of HPV infection based on the measurement and analysis of gene expression levels. In particular, WO 99/29890 describes methods which are based on measuring the levels of expression of two or more HPV genes (e.g. HPV E6, E7, L1 and E2) and then comparing the ratio of expression of combinations of these genes to provide an indication of the stage of HPV-based disease in a patient.

10           The present inventors have determined that it is possible to make a clinically useful assessment of HPV-associated disease based only on a simple positive/negative determination of expression of HPV L1 and E6 mRNA transcripts, with no requirement for accurate quantitative measurements of expression levels or for determination of differences in the levels of expression of the two transcripts. This method is technically simple and, in a preferred embodiment, is amenable to automation in a mid-to-high throughput format. Furthermore, on the basis of results obtained using the method of the invention the inventors have defined a novel scheme for classification of patients on the basis of risk of developing cervical carcinoma which is related to disease-relevant molecular changes in the pattern of HPV gene expression and is independent of CIN classification.

30           Therefore, in a first aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or full length E6 mRNA are scored as being at risk of developing cervical carcinoma.

- 7 -

A positive screening result in the method of the invention is indicated by positive expression of L1 mRNA and/or E6 mRNA in cells of the cervix. Positive expression of either one of these mRNAs or both mRNAs is taken as an indication that the subject is "at risk" for development of cervical carcinoma. Women who express E6 mRNA are at high risk of developing cell changes because oncogenic E6 and E7 bind to cell cycle regulatory proteins and act as a switch for cell proliferation. Clear expression of E6 mRNA provides a direct indication of cell changes in the cervix. Expression of L1 mRNA, with or without expression of E6 mRNA is also indicative of the presence of an active HPV.

In the wider context of cervical screening, women identified as positive for L1 and/or E6 mRNA expression may be selected for further investigation, for example using cytology. Thus, at one level the method of the invention may provide a technical simple means of pre-screening a population of women in order to identify HPV-positive subjects who may be selected for further investigation.

In a specific embodiment, the method of the invention may be used to classify subjects into four different classes of risk for developing cervical carcinoma on the basis of positive/negative scoring of expression of L1 and E6 mRNA.

Accordingly, in a further aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of HPV and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of



- 8 -

risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

5 Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 or 68. Those individuals positive for expression of E6 mRNA from at least one of HPV  
10 types 16, 18, 31 or 33 are scored as being at higher risk, for example in comparison to individuals negative for these types but positive for expression of E6 mRNA from at least one of HPV types 35, 39, 45, 52, 56, 58, 59, 66 or 68.

15 Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 or 68. Those individuals positive  
20 for expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33 are scored as being at higher risk, for example in comparison to individuals negative for these types but positive for expression of E6 mRNA from at least one of HPV types 35, 39, 45, 52, 56, 58, 59, 66 or 68.

25 Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA from the cancer-associated HPV types, (e.g. negative for expression of E6 mRNA from HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68).

30 Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

35

In a preferred embodiment, positive expression is indicated by the presence of more than 50 copies of

- 9 -

the transcript per ml (or total volume of the sample) and negative expression is indicated by the presence of less than 1 copy of the transcript per ml (or total volume of the sample).

5

The above classification is based on molecular events which are relevant to risk of developing cervical carcinoma and is independent of the CIN status of the subjects. Thus, this method of classification may provide an alternative to the use of cytology in the routine screening of women to identify those at potential risk of developing cervical carcinoma. The method may also be used as an adjunct to cytology, for example as a confirmatory test to confirm a risk assessment made on the basis of cytology.

Women positive for expression of high risk E6 mRNA from one of HPV types 16, 18, 31 or 33 but negative for expression of L1 are in the highest level of risk of developing severe cell changes and cell abnormalities. This is due to the fact that a negative result for L1 mRNA expression is directly indicative of integrated HPV, and therefore a higher probability of high and constant expression of E6 and E7. Integration of a virus in the human genome has also a direct impact on the stability of the cells. Integration of HPV also reduces the possibility of regression of cell changes.

30

Women positive for expression of E6 mRNA from one of HPV types 16, 18, 31 or 33 and positive for expression of L1 mRNA have a "high risk" HPV expression and it is still possible that the HPV has been integrated. However, the risk of these women is not classed as high as those who are L1 negative and E6 positive, since there is a reasonable probability

35

- 10 -

that they do not have integrated HPV.

Women negative for expression of E6 mRNA from HPV types 16, 18, 31 or 33 but positive for expression of E6 mRNA from another HPV type, e.g. 35, 39, 45, 52, 56, 58, 59, 66 and 68, are still considered "at risk" and may therefore be placed in risk categories 1 or 2 (as defined above) depending on whether they are positive or negative for expression of L1 mRNA.

10

Women positive for L1 mRNA but negative for E6 mRNA are scored as being at moderate risk. There may be high-risk HPV types in the sample and L1 expression is indicative of lytic activity. There may also be integrated HPV types but only with viruses that are rare. However, detection of lytic activity may show that the cell may soon develop some changes.

15

In the wider context of cervical screening the method of the invention may be used to classify women according to risk of developing cervical carcinoma and therefore provide a basis for decisions concerning treatment and/or further screening. By way of example: women in risk category 1, particularly those who exhibit positive expression of E6 mRNA from at least one of HPV types 16, 18, 31 or 33, might be identified as requiring "immediate action", meaning conisation or colposcopy, including a biopsy and histology.

25

30

Women in risk category 2, as defined above, might be scored as requiring immediate attention, meaning colposcopy alone or colposcopy including a biopsy and histology.

35

Women in risk category 3, as defined above, might be scored as requiring immediate re-test, meaning

- 11 -

recall for a further test for HPV expression immediately or after a relatively short interval, e.g. six months.

- 5           Women in risk category 4, as defined above, might be returned to the screening program, to be re-tested for HPV expression at a later date.

10           In a further embodiment the invention provides an *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein  
15           subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV.

20           The term "integrated HPV" refers to an HPV genome which is integrated into the human genome.

25           The term "modified episomal HPV genome" is taken to mean an HPV genome which is retained within a cell of the human subject as an episome, i.e. not integrated into the human genome, and which carries a modification as compared to the equivalent wild-type HPV genome, which modification leads to constitutive or persistent expression of transcripts of the E6 and/or E7 genes. The "modification" will typically be  
30           a deletion, a multimerisation or concatemerisation of the episome, a re-arrangement of the episome etc affecting the regulation of E6/E7 expression.

35           As aforesaid, the presence of integrated HPV or a modified episomal HPV genome is indicated by a negative result for L1 mRNA expression, together with

- 12 -

a positive result for expression of E6 mRNA in cells of the cervix. Therefore, the ability to predict the presence of integrated HPV or a modified episomal HPV genome in this assay is critically dependent on the ability to score a negative result for L1 mRNA expression. This requires a detection technique which has maximal sensitivity, yet produces minimal false-negative results. In a preferred embodiment this is achieved by using a sensitive amplification and real-time detection technique to screen for the presence or absence of L1 mRNA. The most preferred technique is real-time NASBA amplification using molecular beacons probes, as described by Leone et al., Nucleic Acids Research., 1998, Vol 26, 2150-2155. Due to the sensitivity of this technique the occurrence of false-negative results is minimised and a result of "negative L1 expression" can be scored with greater confidence.

In a further embodiment, a method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome may be based on screening for expression of E6 mRNA alone. Thus, the invention relates to an *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises screening the subject for expression of mRNA transcripts from the E6 gene of human papillomavirus, wherein subjects positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome.

Moreover, individuals may be sorted into one of two categories of risk for development of cervical carcinoma based on an "on/off" determination of expression of E6 mRNA alone. Therefore, the invention provides an *in vitro* method of screening human

- 13 -

subjects to assess their risk of developing cervical carcinoma, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV and sorting the subject into one of two categories of risk for development of cervical carcinoma based on expression of E6 mRNA, wherein individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified as "high risk" for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV or a modified episomal HPV genome and are therefore classified as "no detectable risk" for development of cervical carcinoma.

Subjects are sorted into one of two categories of risk for development of cervical carcinoma based on an "on/off" determination of expression of E6 mRNA in cells of the cervix. Individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified "high risk" for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV a modified episomal HPV genome and are therefore classified as "no detectable risk" for development of cervical carcinoma.

In the context of cervical screening classification of subjects into the two groups having "high risk" or "no detectable risk" for development of cervical carcinoma provides a basis for decisions concerning treatment and/or further screening. For example subjects in the high risk category may be scored as requiring immediate further analysis, e.g. by histological colposcopy, whilst those in the no

- 14 -

detectable risk category may be referred back to the screening program at three or five year intervals. These methods are particularly useful for assessing risk of developing carcinoma in subjects known to be  
5 infected with HPV, e.g. those testing positive for HPV DNA, or subjects who have previously manifested a cervical abnormality via cytology or pap smear. Subjects placed in the "no detectable risk" category on the basis of E6 mRNA expression may have HPV DNA  
10 present but the negative result for E6 expression indicates that HPV is unrelated to oncogene activity at the time of testing.

The presence of integrated HPV or a modified  
15 episomal HPV genome, as indicated by a positive result for E6 mRNA expression, is itself indicative that the subject has abnormal cell changes in the cervix. Therefore, the invention also relates to an in vitro method of identifying human subjects having abnormal  
20 cell changes in the cervix, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV, wherein individuals positive for expression of E6 mRNA are identified as having abnormal cell changes in the cervix.

25  
The term "abnormal cell changes in the cervix" encompasses cell changes which are characteristic of more severe disease than low-grade cervical lesions or low squamous intraepithelial lesions, includes cell  
30 changes which are characteristic of disease of equal or greater severity than high-grade CIN (defined as a neoplastic expansion of transformed cells), CIN (cervical intraepithelial neoplasia) III, or high squamous intraepithelial neoplasia (HSIL), including  
35 lesions with multiploid DNA profile and "malignant" CIN lesions with increased mean DNA-index values, high percentage of DNA-aneuploidy and 2.5c Exceeding Rates

- 15 -

(Hanselaar *et al.*, 1992, *Anal Cell Pathol.*, 4:315-324; Rihet *et al.*, 1996, *J. Clin Pathol* 49:892-896; and McDermott *et al.*, 1997, *Br. J. Obstet Gynaecol.* 104:623-625).

5

Cervical Intraepithelial Neoplasia (abbreviated "CIN"), also called Cervical Dysplasia, is a cervical condition caused Human Papilloma Virus. CIN is classified as I, II or III depending on its severity. It is considered a pre-cancerous abnormality, but not an actual cancer. The mildest form, CIN I, usually goes away on its own, although rarely it can progress to cancer. The more severe forms, CIN II and CIN III, most often stay the same or get worse with time. They can become a cancer, but almost never do if treated adequately.

15

HPV has been identified as a causative agent in development of cellular changes in the cervix, which may lead to the development of cervical carcinoma. These cellular changes are associated with constitutive or persistent expression of E6/E7 proteins from the HPV viral genome. Thus, it is possible to conclude that subjects in which expression of E6 mRNA can be detected, particularly those subjects who exhibit persistent E6 expression when assessed over a period of time, already manifest cellular changes in the cervix. These changes may have taken place in only a very few cells of the cervix, and may not be detectable by conventional cytology. Nevertheless, with the use of sensitive, specific and accurate methods for detection of E6 mRNA it is possible to identify those subjects who already exhibit cellular changes in the cervix at a much earlier stage than would be possible using conventional cytological screening. This will allow earlier intervention with treatments aimed at

20

25

30

35



- 16 -

preventing the development of cervical carcinoma.

As a result of HPV integration into the human genome or as a result of the "modification" in a modified episomal HPV genome, normal control of the viral E6/E7 oncogene transcription is lost (Durst et al., 1985, J Gen Virol, 66(Pt 7): 1515-1522; Pater and Pater, 1985 Virology 145:313-318; Schwarz et al., 1985, Nature 314: 111-114; Park et al., 1997, *ibid*). In contrast, in premalignant lesions and HPV-infected normal epithelium papillomaviruses predominate in "unmodified" episomal forms, hence oncogene (E6/E7) transcription may be absent or efficiently down-regulated (Johnson et al., 1990, J Gen Virol, 71(Pt 7): 1473-1479; Falcinelli et al., 1993, J Med Virol, 40: 261-265). Integration of human papillomavirus type 16 DNA into the human genome is observed to lead to a more unstable cell activity/genome, and increased stability of E6 and E7 mRNAs (Jeon and Lambert, 1995, Proc Natl Acad Sci USA 92: 1654-1658). Thus HPV integration, typically found in cervical cancers but only infrequently found in CIN lesions (Carmody et al., 1996, Mol Cell Probes, 10: 107-116), appears to be an important event in cervical carcinogenesis.

The present methods detect E6/E7 viral mRNA expression in the cervix instead of DNA. E6/E7 viral expression in cervical cells is a much more accurate assessment of the risk of developing cancer than simply showing that the HPV virus is present. Furthermore, the detection of HPV oncogene transcripts may be a more sensitive indicator of the direct involvement of viral oncogenes in carcinogenesis (Rose et al., 1994, Gynecol Oncol, 52: 212-217; Rose et al., 1995, Gynecol Oncol, 56: 239-244). Detection of E6/E7

- 17 -

transcripts by amplification and detection is a useful diagnostic tool for risk evaluations regarding the development of CIN and its progression to cervical cancer, especially in high-risk HPV type-infected patients with ASCUS and CIN I (Sotlar et al., 1998, Gynecol Oncol, 69: 114-121; Selinka et al., 1998, Lab Invest, 78: 9-18).

The expression of E6/E7 transcripts of HPV-16/18 is uniformly correlated with the physical status of HPV DNAs (Park et al., 1997, Gynecol Oncol, Vol:65(1), 121-9). In most cervical carcinoma cells the E6 and E7 genes of specific human papillomaviruses are transcribed from viral sequences integrated into host cell chromosomes (von Kleben Doeberitz et al., 1991, Proc Natl Acad Sci U S A. Vol:88(4), 1411-5). Viral load and integration has been evaluated in a large series of CIN lesions (Pietsaro et al., 2002, J Clin Microbiol, Vol:40(3), 886-91). Only one sample contained exclusively episomal HPV16 DNA, and this lesion regressed spontaneously. Seventeen of 37 invasive cervical carcinoma samples were identified previously as containing the completely integrated HPV16 genome by using PCR covering the entire E1/E2 gene, and this was confirmed by rliPCR in 16 cases. One case, however, showed a low level of episomal deoxyribonucleic acid in addition to the predominant integrated form. Of the remaining 20 carcinoma samples showing episomal forms in the previous analysis, 14 were found to contain integrated forms using rliPCR, and four contained multimeric (modified) episomal forms. Thus, in total, 31 of 37 of the carcinomas (84%) showed integrated HPV16 genome, while absence of integration could not be detected. (Kalantari et al., 2001, Diagn Mol Pathol, Vol:10(1), 46-54).

- 18 -

There have been virtually no observations that cervical carcinoma cells exist without integrated HPV or modified episomal HPV DNA (Kalantari et al. 2001; Pietsaro et al., 2002, *ibid*). It has further been shown that E6 and E7 may only be transcribed from integrated or modified episomal HPV DNA (von Kleben Doeberitz et al., 1991, *ibid*). Therefore, the inventors surmise that detection of E6/E7 expression provides a direct indication of integrated HPV or modified episomal HPV and high oncogene activity, and conclude that in a clinical context detection of E6 (E6/E7) expression alone is sufficient to identify subjects at "high risk" of developing cervical carcinoma. In other words, if E6/E7 mRNA expression can be detected in a cervical sample, this is directly indicative of cellular abnormalities in the cervix and there is a very high risk of development of cervical carcinoma due to persistent HPV oncogene activity. Therefore, detection of E6/E7 mRNA in a human subject indicates that the subject has a very high risk of developing cervical carcinoma and should undergo immediate further screening, e.g. by colposcopy.

If HPV E6/E7 mRNA expression is not detected, the subject may still have an HPV infection. However due to absence of integration and oncogene activity, it may regress spontaneously (as observed by Pietsaro et al., 2002, *ibid*).

In a clinical context the performance of methods which rely on screening for expression of E6 mRNA alone is critically dependent on the ability to score a negative result for E6 mRNA expression with confidence. This again requires a detection technique which has maximal sensitivity, yet produces minimal false-negative results. In a preferred embodiment

- 19 -

this is achieved by using a sensitive amplification and real-time detection technique to screen for the presence or absence of E6 mRNA. The most preferred technique is real-time NASBA amplification using  
5 molecular beacons probes, as described by Leone et al., Nucleic Acids Research., 1998, Vol 26, 2150-2155. Due to the sensitivity of this technique the occurrence of false-negative results is minimised and a result of "negative E6 expression" can be scored  
10 with greater confidence. This is extremely important if the assays are to be used in the context of a clinical screening program.

In the methods based on detection of E6 mRNA  
15 alone it is preferred to detect at least types HPV 16, 18, 31, 33 and 45, and in a preferred embodiment the assay may detect only these HPV types. DNA from HPV types 16, 18, 31 and 33 has been detected in more than 87% of cervical carcinoma samples (Karlsen et al.,  
20 1996, J Clin Microbiol, 34:2095-2100). Other studies have shown that E6 and E7 are almost invariably retained in cervical cancers, as their expression is likely to be necessary for conversion to and maintenance of the malignant state (Choo et al., 1987,  
25 J Med Virol 21:101-107; Durst et al., 1995, Cancer Genet Cytogenet, 85: 105-112). In contrast to HPV detection systems which are based on detection of the undamaged genome or the L1 gene sequence, detection of HPV mRNA expressed from the E6/E7 area may detect more  
30 than 90% of the patients directly related to a risk of developing cervical carcinoma.

In the clinic, methods based on detection of E6 mRNA are preferred for use in post-screening, i.e.  
35 further analysis of individuals having a previous diagnosis of ASCUS, CIN 1 or Condyloma. The method may be used to select those with a high risk of

- 20 -

developing cervical carcinoma from amongst the group of individuals having a previous diagnosis of ASCUS, CIN 1 or Condyloma. ASCUS, Condyloma and CIN I may be defined as more or less the same diagnosis due to very low reproducibility between different cytologists and different cytological departments. Östör (Int J. Gyn Path. 12:186-192. 1993) found that only around 1% of the CIN 1 cases may progress to cervical carcinoma. Thus, there is a genuine need for an efficient method of identifying the subset of individuals with ASCUS, Condyloma or CIN I who are at substantial risk of developing cervical carcinoma. One of HPV types 16, 18, 31 or 33 was detected in 87% of the cervical carcinoma cases study by Karlsen et al., 1996. By inclusion of HPV 45, nearly 90% of the cervical carcinoma samples are found to be related to these five HPV types. Therefore, calculated from the data provided by Östör (Int J. Gyn Path. 12:186-192. 1993) more than 99.9% are detected cases with ASCUS, CIN I or condyloma are missed by our HPV-Proofer kit.

In the methods of the invention "positive expression" of an mRNA is taken to mean expression above background. There is no absolute requirement for accurate quantitative determination of the level of mRNA expression or for accurate determination of the relative levels of expression of L1 and E6 mRNA.

In certain embodiments, the methods of the invention may comprise a quantitative determination of levels of mRNA expression. In a preferred embodiment in order to provide a clear distinction between "positive expression" and "negative expression" a determination of "positive expression" may require the presence of more than 50 copies of the relevant mRNA (per ml of sample or per total volume of sample), whereas a determination of "negative expression" may

- 21 -

require the presence of less than 1 copy of the relevant mRNA (per ml of sample or per total volume of sample).

5           The methods of the invention will preferably involve screening for E6 mRNA using a technique which is able to detect specifically E6 mRNA from cancer-associated HPV types, more preferably "high risk" cancer-associated HPV types. In the most preferred  
10           embodiment the methods involve screening for E6 mRNA using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31 and 33, and preferably also 45. Most preferably, the method will specifically detect expression of E6 mRNA from at least one of HPV types  
15           16, 18, 31, 33, and preferably also 45, and most preferably all five types. However, women positive for positive for expression of E6 from other types than 16, 18, 31, 33 and 45, e.g. 35, 39, 45, 52, 56, 58, 59, 66 and 68 may still be "at risk" of developing  
20           cervical carcinoma. Thus, the method may encompass screening for expression of E6 mRNA from one or more of these HPV types, most preferably in addition to screening for E6 mRNA from HPV types 16, 18, 31, 33 and 45. Certain HPV types exhibit a marked  
25           geographical/population distribution. Therefore, it may be appropriate to include primers specific for an HPV type known to be prevalent in the population/geographical area under test, for example in addition to screening for HPV types 16, 18, 31, 33  
30           and 45.

          For the avoidance of doubt, unless otherwise stated the term "E6 mRNA" as used herein encompasses all naturally occurring mRNA transcripts which contain  
35           all or part of the E6 open reading frame, including naturally occurring splice variants, and therefore includes transcripts which additionally contain all or

- 22 -

part of the E7 open reading frame (and indeed further open reading frames). The terms "E6/E7 mRNA", "E6/E7 transcripts" etc are used interchangeably with the terms "E6 mRNA", "E6 transcripts" and also encompass naturally occurring mRNA transcripts which contain all or part of the E6 open reading frame, including naturally occurring splice variants, and transcripts which contain all or part of the E7 open reading frame. The term "oncogene expression", unless otherwise stated, also refers to naturally occurring mRNA transcripts which contain all or part of the E6 open reading frame, including naturally occurring splice variants, and transcripts which contain all or part of the E7 open reading frame.

Four E6/E7 mRNA species have so far been described in cells infected with HPV 16, namely an unspliced E6 transcript and three spliced transcripts denoted E6\*I, E6\*II and E6\*III (Smotkin D, et al., J Virol. 1989 Mar 63(3):1441-7; Smotkin D, Wettstein FO. Proc Natl Acad Sci USA. 1986 Jul 83(13):4680-4; Doorbar J. et al., Virology. 1990 Sep 178(1):254-62; Cornelissen MT, et al. J Gen Virol. 1990 May 71(Pt 5):1243-6; Johnson MA, et al. J Gen Virol. 1990 Jul 71(Pt 7):1473-9; Schneider-Maunoury S, et al. J Virol. 1987 Oct 61(10):3295-8; Sherman L, et al. Int J Cancer. 1992 Feb 50(3):356-64). All four transcripts are transcribed from a single promoter (p97) located just upstream of the second ATG of the E6 ORF.

In one embodiment the methods may comprise screening for E6 transcripts which contain all or part of the E7 open reading frame, This may be accomplished, for example, using primers or probes specific for the E7 coding region.

In a further embodiment, the methods may comprise

- 23 -

screening for the presence of "full length" E6 transcripts. In the case of HPV 16 the term "full length E6 transcripts" refers to transcripts which contain all of the region from nucleotide (nt) 97 to  
5 nt 880 in the E6 ORF, inclusive of nt 97 and 880. Nucleotide positions are numbered according to standard HPV nomenclature (see Human Papillomavirus Compendium OnLine, available via the internet or in  
10 paper form from HV Database, Mail Stop K710, Los Alamos National Laboratory, Los Alamos, NM 87545, USA). Specific detection of full length transcripts may be accomplished, for example, using primers or probes which are specific for the region which is present only in full length E6 transcripts, not in  
15 splice variants. Different HPV types exhibit different patterns of E6/E7 mRNA expression. Transcript maps for various HPV types, including HPV types 16 and 31, which may be used to assist in the design of probes or primers for detection of E6/E7  
20 transcripts are publicly available via the Human Papillomavirus Compendium (as above).

E6 oligonucleotide primers are described herein which are suitable for use in amplification of regions  
25 of the E6 mRNA from various HPV types by NASBA or PCR.

In a preferred embodiment methods which involve screening for L1 mRNA expression may comprise screening for L1 mRNA expression using a technique  
30 which is able to detect L1 mRNA from substantially all known HPV types or at least the major cancer-associated HPV types (e.g. preferably all of HPV types 16, 18, 31 and 33) . L1 primers and probes are described herein which are capable of detecting L1  
35 mRNA from HPV types 6, 11, 16, 18, 31, 33, 35 and 51 in cervical samples.



- 24 -

Detection of L1 transcripts can be said to detect HPV "virulence", meaning the presence of HPV lytic activity. Detection of E6/E7 transcripts can be said to detect HPV "pathogenesis" since expression of these  
5 mRNAs is indicative of molecular events associated with risk of developing carcinoma.

In a study of 4589 women it was possible to detect all except one case of CIN III lesions or  
10 cancer using a method based on screening for expression of E6 and L1 mRNA (see accompanying Examples).

In further embodiments, the above-described  
15 methods of the invention may comprise screening for expression of mRNA transcripts from the human p16<sup>ink4a</sup> gene, in addition to screening for expression of HPV L1 and/or E6 transcripts.

20 A positive result for expression of p16<sup>ink4a</sup> mRNA is taken as a further indication of risk of developing cervical carcinoma.

P16<sup>ink4a</sup>, and the related family members, may  
25 function to regulate the phosphorylation and the growth suppressive activity of the retinoblastoma gene product (RB). In support of this, it has been found that there is an inverse relationship between the expression of p16<sup>ink4a</sup> protein and the presence of  
30 normal RB in selected cancer cell lines; p16<sup>ink4a</sup> protein is detectable when RB is mutant, deleted, or inactivated, and it is markedly reduced or absent in cell lines that contain a normal RB. Kheif et al. (Kheif SN et al., Proc. Natl. Acad. Sci. USA  
35 93:4350-4354. 1996), found that p16<sup>ink4a</sup> protein is expressed in human cervical carcinoma cells that contain either a mutant RB or a wild-type RB that is

- 25 -

functionally inactivated by E7. They also show that the inactivation of RB correlates with an upregulation of p16<sup>ink4a</sup> confirming a feedback loop involving p16<sup>ink4a</sup> and RB. Milde-Langosch et al. (Milde-Langosch K, et al., (2001) Virchows Arch 439: 55-61) found that there were significant correlations between strong p16 expression and HPV16/18 infection and between strong p16 expression and HPV 16/18 E6/E7 oncogene expression. Klaes et al., (Klaes R, et al., (2001) Int J Cancer 92: 276-284) observed a strong over expression of the p16<sup>ink4a</sup> gene product in 150 of 152 high-grade dysplastic cervical lesions (CIN II to invasive cancer), whereas normal cervical epithelium or inflammatory or metaplastic lesions were not stained with the p16<sup>ink4a</sup> specific monoclonal antibody E6H4. All CIN I scored lesions associated with LR-HPV types displayed no or only focal or sporadic reactivity, whereas all but two CIN I scored lesions associated with HR-HPV types showed strong and diffuse staining for p16<sup>ink4a</sup>.

The disclosed screening methods may be carried out on a preparation of nucleic acid isolated from a clinical sample or biopsy containing cervical cells taken from the subject under test. Suitable samples which may be used as a source of nucleic acid include (but not exclusively) cervical swabs, cervical biopsies, cervical scrapings, skin biopsies/warts, also paraffin embedded tissues, and formalin or methanol fixed cells.

The preparation of nucleic acid to be screened using the disclosed method must include mRNA, however it need not be a preparation of purified poly A+ mRNA and preparations of total RNA or crude preparations of total nucleic acid containing both RNA and genomic DNA, or even crude cell lysates are also suitable as

- 26 -

starting material for a NASBA reaction. Essentially any technique known in the art for the isolation of a preparation of nucleic acid including mRNA may be used to isolate nucleic acid from a test sample. A  
5 preferred technique is the "Boom" isolation method described in US-A-5,234,809 and EP-B-0389,063. This method, which can be used to isolate a nucleic acid preparation containing both RNA and DNA, is based on the nucleic acid binding properties of silicon dioxide  
10 particles in the presence of the chaotropic agent guanidine thiocyanate (GuSCN).

The methods of the invention are based on assessment of active transcription of the HPV genome  
15 in cervical cells. The methods are not limited with respect to the precise technique used to detect mRNA expression. Many techniques for detection of specific mRNA sequences are known in the art and may be used in accordance with the invention. For example, specific  
20 mRNAs may be detected by hybridisation, amplification or sequencing techniques.

It is most preferred to detect mRNA expression by means of an amplification technique, most preferably  
25 an isothermal amplification such as NASBA, transcription-mediated amplification, signal-mediated amplification of RNA technology, isothermal solution phase amplification, etc. All of these methods are well known in the art. More preferably mRNA expression  
30 is detected by an isothermal amplification in combination with real-time detection of the amplification product. The most preferred combination is amplification by NASBA, coupled with real-time detection of the amplification product using molecular  
35 beacons technology, as described by Leone et al., Nucleic Acids Research, 1998, Vol 26, 2150-2155.

- 28 -

time NASBA" which allows continuous monitoring of the formation of the product of the NASBA reaction over the course of the reaction. In a preferred embodiment this may be achieved using a "molecular beacons" probe comprising an HPV-specific sequence capable of annealing to the NASBA product, a stem-duplex forming oligonucleotide sequence and a pair of fluorescer/quencher moieties, as known in the art and described herein. If the molecular beacons probe is added to the reaction mixture prior to amplification it may be possible to monitor the formation of the NASBA product in real-time (Leone et al., Nucleic Acids Research, 1998, Vol 26, 2150-2155). Reagent kits and instrumentation for performing real-time NASBA detection are available commercially (e.g. NucliSens™ EasyQ system, from Organon Teknika).

In a further approach, the molecular beacons technology may be incorporated into the primer 2 oligonucleotide allowing real-time monitoring of the NASBA reaction without the need for a separate hybridisation probe.

In a still further approach the products of the NASBA reaction may be monitored using a generic labelled detection probe which hybridises to a nucleotide sequence in the 5' terminus of the primer 2 oligonucleotide. This is equivalent to the "NucliSens™" detection system supplied by Organon Teknika. In this system specificity for NASBA products derived from the target HPV mRNA may be conferred by using HPV-specific capture probes comprising probe oligonucleotides as described herein attached to a solid support such as a magnetic microbead. Most preferably the generic labelled detection probe is the ECL™ detection probe supplied by Organon Teknika. NASBA amplicons are hybridized to

- 29 -

the HPV-specific capture probes and the generic ECL probe (via a complementary sequence on primer 2). Following hybridization the bead/amplicon/ECL probe complexes may be captured at the magnet electrode of an automatic ECL reader (e.g. the NucliSens™ reader supplied by Organon Teknika). Subsequently, a voltage pulse triggers the ECL™ reaction.

The detection of HPV mRNA is also of clinical relevance in cancers other than cervical carcinoma including, for example, head and neck carcinoma, oral and tongue carcinoma, skin carcinoma, anal and vaginal carcinoma. Detection of HPV mRNA may also be very useful in the diagnosis of micrometastases in lymph nodes in the lower part of the body. Hence, the invention also contemplates screens for susceptibility to the above-listed cancers based on screening for expression of HPV L1 and E6 transcripts.

In accordance with a further aspect of the invention there is provided a kit for use in the detection of transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31 and 33, and preferably also HPV 45, and one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31 and 33, and preferably also HPV 45.

"Primer-pair" taken to mean are pair of primers which may be used in combination to amplify a specific region of the L1 or E6 mRNA using any known nucleic acid technique. In preferred embodiments the primer-pairs included in the kit will be suitable for use in NASBA amplification or similar isothermal amplification techniques.

- 30 -

The individual primers making up each primer-pair included in the kit may be supplied separately (e.g. a separate container of each primer) or, more preferably, may be supplied mixed in a single container. Combinations of two or more primer-pairs may be supplied ready-mixed in a single container within the kit. It may be convenient to supply two or more primer-pairs in a single container where the two or more amplification reactions are to be "multiplexed", meaning performed simultaneously in a single reaction vessel.

The primer-pair(s) suitable for use in amplification of a region of E6 transcripts should enable amplification a region of E6 mRNA from at least the major cancer-associated HPV types 16, 18, 31 and 33, and preferably also HPV 45. There are several different ways in which this can be achieved.

In one embodiment, the kit may contain separate primer-pairs specific for each of HPV types 16, 18, 31 and 33, and preferably also HPV 45. These primer-pairs may be supplied within the kit in separate containers, or they may be supplied as mixtures of two or more primer-pairs in a single container, for example to enable multiplexing of the amplification reactions.

In a further embodiment, the kit may contain a single primer-pair capable of amplifying a region of the E6 gene from HPV types 16, 18, 31 and 33, and preferably also HPV 45, which thus enables amplification of all four (preferably five) types in a single amplification reaction. This could, for example, be achieved with the use of a pair of degenerate primers or by selection of a region of the E6 mRNA which is highly conserved across HPV types.

- 31 -

The E6 primer-pair may correspond to any region of the E6 mRNA, and may enable amplification of all or part of the E6 open reading frame and/or the E7 open reading frame.

5

The kit may further include primer-pairs suitable for use in amplification of E6 mRNA from HPV types other than types 16, 18, 31 and 33, and preferably also HPV 45. For example, the kit may be supplemented with E6 primers for detection of an HPV type which is endemic in a particular geographical area or population.

The primer-pair(s) suitable for use in amplification of a region of L1 transcripts should be capable of amplifying a region of L1 mRNA from at least the major cancer-associated HPV types 16, 18, 31 and 33, and preferably also HPV 45, and will preferably be suitable for use in amplification of a region of L1 mRNAs from substantially all known HPV types. With the use of such primers it is possible to test for active transcription of L1 mRNA from multiple HPV types in a single amplification reaction.

It is possible to design primers capable of detecting L1 transcripts from multiple HPV types by selecting regions of the L1 transcript which are highly conserved.

In a further approach, specificity for multiple HPV types may be achieved with the use of degenerate oligonucleotide primers or complex mixtures of polynucleotides which exhibit minor sequence variations, preferably corresponding to sites of sequence variation between HPV genotypes. The rationale behind the use of such degenerate primers or mixtures is that the mixture may contain at least one

- 32 -

primer-pair capable of detecting each HPV type.

5 In a still further approach specificity for multiple HPV types may be achieved by incorporating into the primers one or more inosine nucleotides, preferably at sites of sequence variation between HPV genotypes.

10 The E6 and L1 primer-pairs may be supplied in separate containers within the kit, or the L1 primer-pair(s) may be supplied as a mixture with one or more E6 primer-pairs in a single container.

15 The kits may further comprise one or more probes suitable for use in detection of the products of amplification reactions carried out using the primer-pairs included within the kit. The probe(s) may be supplied as a separate reagent within the kit. Alternatively, the probe(s) may be supplied as a  
20 mixture with one or more primer-pairs.

The primers and probes included in the kit are preferably single stranded DNA molecules. Non-natural synthetic polynucleotides which retain the ability to  
25 base-pair with a complementary nucleic acid molecule may also be used, including synthetic oligonucleotides which incorporate modified bases and synthetic oligonucleotides wherein the links between individual nucleosides include bonds other than phosphodiester  
30 bonds. The primers and probes may be produced according to techniques well known in the art, such as by chemical synthesis using standard apparatus and protocols for oligonucleotide synthesis.

35 The primers and probes will typically be isolated single-stranded polynucleotides of no more than 100 bases in length, more typically less than 55 bases in



- 33 -

length. For the avoidance of doubt it is hereby stated that the terms "primer" and "probe" exclude naturally occurring full-length HPV genomes.

5           Several general types of oligonucleotide primers and probes incorporating HPV-specific sequences may be included in the kit. Typically, such primers and probes may comprise additional, non-HPV sequences, for example sequences which are required for an  
10           amplification reaction or which facilitate detection of the products of the amplification reaction.

          The first type of primers are primer 1 oligonucleotides (also referred to herein as NASBA P1  
15           primers), which are oligonucleotides of generally approximately 50 bases in length, containing an average of about 20 bases at the 3' end that are complementary to a region of the target mRNA. Oligonucleotides suitable for use as NASBA P1 primers  
20           are denoted "P1/PCR" in Table 1. P1 primer oligonucleotides have the general structure  $X_1$ -SEQ, wherein SEQ represents an HPV-specific sequence and  $X_1$  is a sequence comprising a promoter that is recognized by a specific RNA polymerase. Bacteriophage  
25           promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the oligonucleotides of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. In a preferred  
30           embodiment, sequence " $X_1$ " may comprise the sequence AATTCTAATACGACTCACTATAGGG or the sequence AATTCTAATACGACTCACTATAGGGAGAAGG. These sequences contains a T7 promoter, including the transcription initiation site for T7 RNA polymerase.  
35           The HPV-specific sequences in the primers denoted in Table 1 as "P1/PCR" may also be adapted for use in standard PCR primers. When these sequences are used

- 34 -

as the basis of NASBA P1 primers they have the general structure  $X_1$ -SEQ, as defined above. The promoter sequence  $X_1$  is essential in a NASBA P1 primer. However, when the same sequences are used as the basis of standard PCR primers it is not necessary to include  $X_1$ .

A second type of primers are NASBA primer 2 oligonucleotides (also referred to herein as NASBA P2 primers) which generally comprise a sequence of approximately 20 bases substantially identical to a region of the target mRNA. The oligonucleotide sequences denoted in Table 1 as "P2/PCR" are suitable for use in both NASBA P2 primers and standard PCR primers.

Oligonucleotides intended for use as NASBA P2 primers may, in a particular but non-limiting embodiment, further comprise a sequence of nucleotides at the 5' end which is unrelated to the target mRNA but which is capable of hybridising to a generic detection probe. The detection probe will preferably be labelled, for example with a fluorescent, luminescent or enzymatic label. In one embodiment the detection probe is labelled with a label that permits detection using ECL™ technology, although it will be appreciated that the invention is in no way limited to this particular method of detection. In a preferred embodiment the 5' end of the primer 2 oligonucleotides may comprise the sequence GATGCAAGGTCGCATATGAG. This sequence is capable of hybridising to a generic ECL™ probe commercially available from Organon Teknika having the following structure:

$Ru(bpy)_3^{2+}$ -GAT GCA AGG TCG CAT ATG AG-3'

In a different embodiment the primer 2

- 35 -

oligonucleotide may incorporate "molecular beacons" technology, which is known in the art and described, for example, in WO 95/13399 by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996, to allow for  
5 real-time monitoring of the NASBA reaction.

Target-specific probe oligonucleotides may also be included within the kit. Probe oligonucleotides generally comprise a sequence of approximately 20-25  
10 bases substantially identical to a region of the target mRNA, or the complement thereof. Example HPV-specific oligonucleotide sequences which are suitable for use as probes are denoted "PO" in Table 1. The probe oligonucleotides may be used as target-specific  
15 hybridisation probes for detection of the products of a NASBA or PCR reaction. In this connection the probe oligonucleotides may be coupled to a solid support, such as paramagnetic beads, to form a capture probe (see below). In a preferred embodiment the 5' end of  
20 the probe oligonucleotide may be labelled with biotin. The addition of a biotin label facilitates attachment of the probe to a solid support via a biotin/streptavidin or biotin/avidin linkage.

25 Target-specific probes enabling real-time detection of amplification products may incorporate "molecular beacons" technology which is known in the art and described, for example, by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996 and in WO  
30 95/13399. Example HPV-specific oligonucleotide sequences suitable for use as molecular beacons probes are denoted "MB" in Table 1.

The term "molecular beacons probes" as used  
35 herein is taken to mean molecules having the structure:

- 36 -

 $X_2$ -arm<sub>1</sub>-target-arm<sub>2</sub>-X<sub>3</sub>

wherein "target" represents a target-specific sequence of nucleotides, "X<sub>2</sub>" and "X<sub>3</sub>" represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and "arm<sub>1</sub>" and "arm<sub>2</sub>" represent complementary sequences capable of forming a stem duplex.

Preferred combinations of "arm<sub>1</sub>" and "arm<sub>2</sub>" sequences are as follows, however these are intended to be illustrative rather than limiting to the invention:

cgcatg-SEQ-catgcg  
ccagct-SEQ-agctgg  
cacgc-SEQ-gcgtg  
cgatcg-SEQ-cgatcg  
ccgtcg-SEQ-cgacgg  
cggacc-SEQ-ggtccg  
ccgaagg-SEQ-ccttcgg  
cacgtcg-SEQ-cgacgtg  
cgcagc-SEQ-gctgcg  
ccaagc-SEQ-gcttgg  
ccaagcg-SEQ-cgcttgg  
cccagc-SEQ-gctggg  
ccaaagc-SEQ-gctttgg  
cctgc-SEQ-gcagg  
ccaccc-SEQ-gggtgg  
ccaagcc-SEQ-ggcttgg  
ccagcg-SEQ-cgctgg  
cgcatg-SEQ-catgcg

The use of molecular beacons technology allows for real-time monitoring of amplification reactions,

- 37 -

for example NASBA amplification (see Leone et al.,  
Nucleic Acids Research., 1998, vol: 26, pp 2150-2155).  
The molecular beacons probes generally include  
complementary sequences flanking the HPV-specific  
5 sequence, represented herein by the notation arm<sub>1</sub> and  
arm<sub>2</sub>, which are capable of hybridising to each other  
form a stem duplex structure. The precise sequences  
of arm<sub>1</sub> and arm<sub>2</sub> are not material to the invention,  
except for the requirement that these sequences must  
10 be capable of forming a stem duplex when the probe is  
not bound to a target HPV sequence.

Molecular beacons probes also include a  
fluorescent moiety and a quencher moiety, the  
15 fluorescent and the quencher moieties being  
represented herein by the notation X<sub>2</sub> and X<sub>3</sub>. As will  
be appreciated by the skilled reader, the fluorescer  
and quencher moieties are selected such that the  
quencher moiety is capable of substantially or  
20 completely quenching the fluorescence from the  
fluorescent moiety when the two moieties are in close  
proximity, e.g. when the probe is in the hairpin  
"closed" conformation in the absence of the target  
sequence. Upon binding to the target sequence, the  
25 fluorescent and quencher moieties are held apart such  
that the fluorescence of the fluorescent moiety is no  
longer quenched.

Many examples of suitable pairs of  
30 quencher/fluorescer moieties which may be used in  
accordance with the invention are known in the art  
(see WO 95/13399, Tyagi and Kramer, *ibid*). A broad  
range of fluorophores in many different colours made  
be used, including for example  
35 5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid  
(EDANS), fluorescein, FAM and Texas Red (see Tyagi,  
Bratu and Kramer, 1998, *Nature Biotechnology*, 16, 49-

- 38 -

53. The use of probes labelled with different coloured fluorophores enables "multiplex" detection of two or more different probes in a single reaction vessel. A preferred quencher is

5 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), a non-fluorescent chromophore, which serves as a "universal" quencher for a wide range of fluorophores. The fluorescer and quencher moieties may be covalently attached to the probe in either orientation, either

10 with the fluorescer at or near the 5' end and the quencher at or near the 3' end or vice versa. Protocols for the synthesis of molecular beacon probes are known in the art. A detailed protocol for synthesis is provided in a paper entitled "Molecular

15 Beacons: Hybridization Probes for Detection of Nucleic Acids in Homogenous Solutions" by Sanjay Tyagi et al., Department of Molecular Genetics, Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA, which is available online via the PHRI

20 website (at [www.phri.nyu.edu](http://www.phri.nyu.edu) or [www.molecular-beacons.org](http://www.molecular-beacons.org))

Suitable combinations of the NASBA P1 and NASBA P2 primers may be used to drive a NASBA amplification

25 reaction. In order to drive a NASBA amplification reaction the primer 1 and primer 2 oligonucleotides must be capable of priming synthesis of a double-stranded DNA from a target region of mRNA. For this to occur the primer 1 and primer 2

30 oligonucleotides must comprise target-specific sequences which are complementary to regions of the sense and the antisense strand of the target mRNA, respectively.

35 In the first phase of the NASBA amplification cycle, the so-called "non-cyclic" phase, the primer 1 oligonucleotide anneals to a complementary sequence in

- 39 -

the target mRNA and its 3' end is extended by the action of an RNA-dependent DNA polymerase (e.g. reverse transcriptase) to form a first-strand cDNA synthesis. The RNA strand of the resulting RNA:DNA hybrid is then digested, e.g. by the action of RNaseH, to leave a single stranded DNA. The primer 2 oligonucleotide anneals to a complementary sequence towards the 3' end of this single stranded DNA and its 3' end is extended (by the action of reverse transcriptase), forming a double stranded DNA. RNA polymerase is then able to transcribe multiple RNA copies from the now transcriptionally active promoter sequence within the double-stranded DNA. This RNA transcript, which is antisense to the original target mRNA, can act as a template for a further round of NASBA reactions, with primer 2 annealing to the RNA and priming synthesis of the first cDNA strand and primer 1 priming synthesis of the second cDNA strand. The general principles of the NASBA reaction are well known in the art (see Compton, J. Nature. 350: 91-92).

The target-specific probe oligonucleotides described herein may also be attached to a solid support, such as magnetic microbeads, and used as "capture probes" to immobilise the product of the NASBA amplification reaction (a single stranded RNA). The target-specific "molecular beacons" probes described herein may be used for real-time monitoring of the NASBA reaction.

Kits according to the invention may also including a positive control containing E6 and/or L1 mRNA from a known HPV type. Suitable controls include, for example, nucleic acid extracts prepared from cell lines infected with known HPV types (e.g. HeLa, CaSki).

- 40 -

Kits further may contain internal control amplification primers, e.g. primers specific for human U1A RNA.

5 Kits containing primers (and optionally probes) suitable for use in NASBA amplification may further comprise a mixture of enzymes required for the NASBA reaction, e.g. enzyme mixture containing an RNA directed DNA polymerase (e.g. a reverse  
10 transcriptase), a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA (e.g. RNaseH) and an RNA polymerase. The RNA polymerase should be one which recognises the promoter sequence present in the 5'  
15 terminal region of the NASBA P1 primers supplied in the reagent kit. The kit may also comprise a supply of NASBA buffer containing the ribonucleosides and deoxyribonucleosides required for RNA and DNA  
20 synthesis. The composition of a standard NASBA reaction buffer will be well known to those skilled in the art (see also Leone et al., *ibid*).

Table 1: E6-specific sequences for inclusion in NASBA/PCR primers and probes

25

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
1	P2/PCR	CCACAGGAGCGACCCAGAAAGTTA	16	116
2	P1/PCR	X <sub>1</sub> -ACGTTTGTGTATTGCTGTTC	16	368
3	P2/PCR	CCACAGGAGCGACCCAGAAA	16	116
30 4	P1/PCR	X <sub>1</sub> -GGTTTGTGTATTGCTGTTC	16	368
5	P1/PCR	X <sub>1</sub> -ATTCCCATCTCTATATACTA	16	258
6	P1/PCR	X <sub>1</sub> -TCACGTCGCAGTAACTGT	16	208
7	P1/PCR	X <sub>1</sub> -TTGCTTGACAGTACACACA	16	191
8	P1/PCR	X <sub>1</sub> -TGCAGTACACACATTCTA	16	186
35 9	P1/PCR	X <sub>1</sub> -GCAGTACACACATTCTAA	16	185
10	P2/PCR	ACAGTTATGCACAGAGCT	16	142
11	P2/PCR	ATATTAGAATGTGTGTAC	16	182
12	P2/PCR	TTAGAATGTGTGTACTGC	16	185
13	P2/PCR	GAATGTGTGTACTGCAAG	16	188



	SEQ ID	Primer /probe type	Sequence	HPV Type	nt
	14	PO	ACAGTTATGCACAGAGCT	16	142
	15	PO	ATATTAGAATGTGTGTAC	16	182
	16	PO	TTAGAATGTGTGTACTGC	16	185
	17	PO	GAATGTGTGTACTGCAAG	16	188
5	18	PO	CTTTGCTTTTCGGGATTATGC	16	235
	19	PO	TATGACTTTGCTTTTCGGGA	16	230
	20	MB	X <sub>2</sub> -arm <sub>1</sub> -TATGACTTTGCTTTTCGGGA-arm <sub>2</sub> -X <sub>3</sub>	16	230
	21	P2/PCR	CAGAGGAGGAGGATGAAATAGTA	16	656
10	22	P1/PCR	X <sub>1</sub> -GCACAACCGAAGCGTAGAGTCACAC	16	741
	23	PO	TGGACAAGCAGAACCGGACAGAGC	16	687
	24	P2/PCR	CAGAGGAGGAGGATGAAATAGA	16	656
	25	P1/PCR	X <sub>1</sub> -GCACAACCGAAGCGTAGAGTCA	16	741
	26	PO	AGCAGAACCGGACAGAGCCATTA	16	693
	27	P2/PCR	ACGATGAAATAGATGGAGTT	18	702
15	28	P1/PCR	X <sub>1</sub> -CACGGACACACAAAGGACAG	18	869
	28	PO	AGCCGAACCACAACGTCACA	18	748
	30	P2/PCR	GAAAACGATGAAATAGATGGAG	18	698
	31	P1/PCR	X <sub>1</sub> -ACACCACGGACACACAAAGGACAG	18	869
	32	PO	GAACCACAACGTCACACAATG	18	752
20	33	MB	X <sub>2</sub> -arm <sub>1</sub> -GAACCACAACGTCACACAATG-arm <sub>2</sub> -X <sub>3</sub>	18	752
	34	P2/PCR	TTCCGGTTGACCTTCTATGT	18	651
	35	P1/PCR	X <sub>1</sub> -GGTCGTCTGCTGAGCTTTCT	18	817
	36	P2/PCR	GCAAGACATAGAAATAACCTG	18	179
	37	P1/PCR	X <sub>1</sub> -ACCCAGTGTAGTTAGTT	18	379
25	38	PO	TGCAAGACAGTATTGGAAC	18	207
	39	P2/PCR	GGAAATACCCTACGATGAAC	31	164
	40	P1/PCR	X <sub>1</sub> -GGACACAACGGTCTTTGACA	31	423
	41	PO	ATAGGGACGACACACCACACGGAG	31	268
	42	P2/PCR	GGAAATACCCTACGATGAACTA	31	164
30	43	P1/PCR	X <sub>1</sub> -CTGGACACAACGGTCTTTGACA	31	423
	44	PO	TAGGGACGACACACCACACGGA	31	269
	45	P2/PCR	ACTGACCTCCACTGTTATGA	31	617
	46	P1/PCR	X <sub>1</sub> -TATCTACTTGTGTGCTCTGT	31	766
	47	PO	GACAAGCAGAACCGGACACATC	31	687
35	48	P2/PCR	TGACCTCCACTGTTATGAGCAATT	31	619
	49	P1/PCR	X <sub>1</sub> -TGCGAATATCTACTTGTGTGCTCT GT	31	766
	50	PO	GGACAAGCAGAACCGGACACATCCAA	31	686
	51	MB	X <sub>2</sub> -arm <sub>1</sub> -GGACAAGCAGAACCGGACACATCCAA-arm <sub>2</sub> -X <sub>3</sub>	31	686
	52	P2/PCR	ACTGACCTCCACTGTTAT	31	617
40	53	P1/PCR	X <sub>1</sub> -CACGATTCCAAATGAGCCCAT	31	809
	54	P2/PCR	TATCCTGAACCAACTGACCTAT	33	618
	55	P1/PCR	X <sub>1</sub> -TTGACACATAAACGAACTG	33	763
	56	PO	CAGATGGACAAGCACAACC	33	694
	57	P2/PCR	TCCTGAACCAACTGACCTAT	33	620
45	58	P1/PCR	X <sub>1</sub> -CCCATAAGTAGTTGCTGTAT	33	807
	59	PO	GGACAAGCACAACCAGCCACAGC	33	699
	60	MB	X <sub>2</sub> -arm <sub>1</sub> -GGACAAGCACAACCAGCCACAGC-arm <sub>2</sub> -X <sub>3</sub>	33	699

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
61	P2/PCR	GACCTTTGTGTCCTCAAGAA	33	431
62	P1/PCR	X <sub>1</sub> -AGGTCAGTTGGTTCAGGATA	33	618
63	PO	AGAACTGCACTGTGACGTGT	33	543
64	P2/PCR	ATTACAGCGGAGTGAGGTAT	35	217
65	P1/PCR	X <sub>1</sub> -GTCTTTGCTTTTCAACTGGA	35	442
66	PO	ATAGAGAAGGCCAGCCATAT	35	270
67	P2/PCR	TCAGAGGAGGAGGAAGATACTA	35	655
68	P1/PCR	X <sub>1</sub> -GATTATGCTCTCTGTGAACA	35	844
69	P2/PCR	CCCGAGGCAACTGACCTATA	35	610
70	P1/PCR	X <sub>1</sub> -GTCAATGTGTGTCTCTGTA	35	770
71	PO	GACAAGCAAAACCAGACCTCAA	35	692
72	PO	GACAAGCAAAACCAGACACC	35	692
73	P2/PCR	TTGTGTGAGGTGCTGGAAGAAT	52	144
74	P1/PCR	X <sub>1</sub> -CCCTCTCTTCTAATGTTT	52	358
75	PO	GTGCCTACGCTTTTATCTA	52	296
76	P2/PCR	GTGCCTACGCTTTTATCTA	52	296
77	P1/PCR	X <sub>1</sub> -GGGGTCTCCAACACTCTGAACA	52	507
78	PO	TGCAAACAAGCGATTTCA	52	461
79	P2/PCR	TCAGGCGTTGGAGACATC	58	157
80	P1/PCR	X <sub>1</sub> -AGCAATCGTAAGCACACT	58	301
81	P2/PCR	TCTGTGCATGAAATCGAA	58	173
82	P1/PCR	X <sub>1</sub> -AGCACACTTTACATACTG	58	291
83	PO	TGAAATGCGTTGAATGCA	58	192
84	PO	TTGCAGCGATCTGAGGTATATG	58	218
85	P2/PCR	TACACTGCTGGACAACAT	B(11)	514
86	P1/PCR	X <sub>1</sub> -TCATCTTCTGAGCTGTCT	B(11)	619
87	P2/PCR	TACACTGCTGGACAACATGCA	B(11)	514
88	P1/PCR	X <sub>1</sub> -GTCACATCCACAGCAACAGGTCA	B(11)	693
89	PO	GTAGGGTTACATTGCTATGA	B(11)	590
90	PO	GTAGGGTTACATTGCTATGAGC	B(11)	590
91	P2/PCR	TGACCTGTTGTGTGGATGTGA	B(11)	693
92	P1/PCR	X <sub>1</sub> -TACCTGAATCGTCCGCCAT	B(11)	832
93	PO	ATWGTGTGTCCCATCTGC	B(11)	794
94	P2/PCR	CATGCCATAAATGTATAGA	C(18)	295
95	P1/PCR	X <sub>1</sub> -CACCGCAGGCACCTTATTAA	C(18) 39 45	408
96	PO	AGAATTAGAGAATTAAGA	C(18) 39 45	324
97	P2/PCR	GCAGACGACCACTACAGCAA	39	210
98	P1/PCR	X <sub>1</sub> -ACACCGAGTCCGAGTAATA	39	344
99	PO	ATAGGGACGGGGAACCACT	39	273
100	P2/PCR	TATTACTCGGACTCGGTGT	39	344
101	P1/PCR	X <sub>1</sub> -CTTGGGTTTCTCTTCGTGTTA	39	558
102	PO	GGACCACAAAACGGGAGGAC	39	531
103	P2/PCR	GAAATAGATGAACCCGACCA	39	703
104	P1/PCR	X <sub>1</sub> -GCACACCACGGACACACAA	39	886
105	PO	TAGCCAGACGGGATGAACCACAGC	39	749
106	P2/PCR	AACCATTGAACCCAGCAGAAA	45	430

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
107	P1/PCR	X <sub>1</sub> -TCTTTCTTGCCGTGCCTGGTCA	45	527
108	PO	GTACCGAGGGCAGTGTATA	45	500
109	P2/PCR	AACCATTAACCCAGCAGAAA	45	430
110	P1/PCR	X <sub>1</sub> -TCTTTCTTGCCGTGCCTGGTCA	45	527
111	P2/PCR	GAAACCATTAACCCAGCAGAAA	45	428
112	P1/PCR	X <sub>1</sub> -TTGCTATACTTGTGTTTCCCTACG	45	558
113	PO	GTACCGAGGGCAGTGTATA	45	500
114	PO	GGACAAACGAAGATTTTACA	45	467
115	P2/PCR	GTTGACCTGTTGTGTTACCAGCAAT	45	656
116	P1/PCR	X <sub>1</sub> -CACCACGGACACACAAAGGACAAG	45	868
117	P2/PCR	CTGTTGACCTGTTGTGTTACGA	45	654
118	P1/PCR	X <sub>1</sub> -CCACGGACACACAAAGGACAAG	45	868
119	P2/PCR	GTTGACCTGTTGTGTTACGA	45	656
120	P1/PCR	X <sub>1</sub> -ACGGACACACAAAGGACAAG	45	868
121	PO	GAGTCAGAGGAGGAAAACGATG	45	686
122	PO	AGGAAAACGATGAAGCAGATGGAGT	45	696
123	PO	ACAACCTACCAGCCGACGAGCCGAA	45	730
124	P2/PCR	GGAGGAGGATGAAGTAGATA	51	658
125	P1/PCR	X <sub>1</sub> -GCCCATTAACTCTGCTGTA	51	807
126	P2/PCR	AGAGGAGGAGGATGAAGTAGATA	51	655
127	P1/PCR	X <sub>1</sub> -ACGGGCAAACCCAGGCTTAGT	51	829
128	PO	GCAGGTGTTCAAGTGTAGTA	51	747
129	PO	TGGCAGTGGAAAGCAGTGGAGACA	51	771
130	P2/PCR	TTGGGGTGTCTGGAGACAAACATCT	56	519
131	P1/PCR	X <sub>1</sub> -TTCATCCTCATCTCATCTCTGA	56	665
132	P2/PCR	TGGGGTGTCTGGAGACAAACATC	56	520
133	P1/PCR	X <sub>1</sub> -CATCCTCATCTCATCTCTGA	56	665
134	P2/PCR	TTGGGGTGTCTGGAGACAAACAT	56	519
135	P1/PCR	X <sub>1</sub> -CCACAACTTACACTCACAACA	56	764
136	PO	AAAGTACCAACGCTGCAAGACGT	56	581
137	PO	AGAATAACACCTCAAACAGAAAT	56	610
138	PO	AGTACCAACGCTGCAAGACGTT	56	583
139	P1/PCR	X <sub>1</sub> -TTGGACAGCTCAGAGGATGAGG	56	656
140	P2/PCR	GATTTTCCTTATGCAGTGTG	56	279
141	P1/PCR	X <sub>1</sub> -GACATCTGTAGCACCTTATT	56	410
142	PO	GACTATTCAGTGTATGGAGC	56	348
143	PO	CAACTGAYCTMYACTGTTATGA	A (16 31 35)	
144	MB	X <sub>2</sub> -arm <sub>1</sub> -CAACTGAYCTMYACTGTTATGA-arm <sub>2</sub> -X <sub>3</sub>	A (16 31 35)	
145	PO	GAAMCAACTGACCTAYWCTGCTAT	A (33 52 58)	
146	MB	X <sub>2</sub> -arm <sub>1</sub> -GAAMCAACTGACCTAYWCTGCTAT-arm <sub>2</sub> -X <sub>3</sub>	A (33 52 58)	
147	PO	AAGACATTATTCAGACTC	C (18 45 39)	
148	MB	X <sub>2</sub> -arm <sub>1</sub> -AAGACATTATTCAGACTC-arm <sub>2</sub> -X <sub>3</sub>	C (18 45 39)	

Table 2: L1-specific sequences for inclusion in  
NASBA/PCR primers and probes

5	SEQ ID	Primer/probe type	Sequence
	149	P2/PCR	AATGGCATTGTGTTGGGGTAA
	150	P1/PCR	X <sub>1</sub> -TCATATTCCTCCCATGTC
	151	PO	TTGTTACTGTTGTTGATACTAC
	152	P2/PCR	AATGGCATTGTGTTGGSRHAA
10	153	P1/PCR	X <sub>1</sub> -TCATATTCCTCMMCATGDC
	154	PO	TTGTTACTGTTGTTGATACYAC
	155	PO	TTGTTACTGTTGTTGATACCAC
	156	P2/PCR	AATGGCATTGTGTTGGSIIAA
	157	P2/PCR	AATGGCATTGTGTTGGIIHAA
15	158	P2/PCR	AATGGCATTGTGTTGGIRIAA
	159	P2/PCR	AATGGCATTGTGTTGGGGTAA
	160	P2/PCR	AATGGCATTGTGTTGGGGAAA
	161	P2/PCR	AATGGCATTGTGTTGGCATAA
	162	P2/PCR	AATGGCATTGTGTTGGGGCAA
20	163	P2/PCR	AATGGCATTGTGTTGGCACAA
	164	P1/PCR	X <sub>1</sub> -TCATATTCCTCMICATGIC
	165	P1/PCR	X <sub>1</sub> -TCATATTCCTCAACATGIC
	166	P1/PCR	X <sub>1</sub> -TCATATTCCTCIICATGTC
	167	P1/PCR	X <sub>1</sub> -TCATATTCCTCIICATGGC
25	168	P1/PCR	X <sub>1</sub> -TCATATTCCTCIICATGAC 3'
	169	P1/PCR	X <sub>1</sub> -TCATATTCCTCIICATGCC 3'

Preferred primers suitable for use in detection  
of HPV L1 and E6 mRNA by NASBA are listed in the  
following tables. However, these are merely  
illustrative and it is not intended that the scope of

- 45 -

the invention should be limited to these specific molecules.

In the following Tables the NASBA P2 primers (p2) include the sequence GATGCAAGGTCGCATATGAG at the 5' end; the NASBA P1 primers (p1) include the sequence AATTCTAATACGACTCACTATAGGGAGAAGG at the 5' end. Oligonucleotides suitable for use as probes are identified by "po". The P2 primers generally contain HPV sequences from the positive strand, whereas the p1 primers generally contain HPV sequences from the negative strand. nt-refers to nucleotide position in the relevant HPV genomic sequence.

Table 3-Preferred E6 NASBA primers and probes

Primer name	Sequence	HPV Type	nt
HAe6701p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAAGTTA	16	116
HAe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGTT TGTTGTATTGCTGTTC	16	368
HAe6702p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAA	16	116
HAe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTTTG TTGTATTGCTGTTC	16	368
HPV16p1	AATTCTAATACGACTCACTATAGGGAGAAGGATTCCC ATCTCTATATACTA	16	258
HAe6702Ap1	AATTCTAATACGACTCACTATAGGGAGAAGGTCA CGTCGCAGTAACTGT	16	208
HAe6702Bp1	AATTCTAATACGACTCACTATAGGGAGAAGGTTG CTTGCACTACACACA	16	191
HAe6702Cp1	AATTCTAATACGACTCACTATAGGGAGAAGGTGC AGTACACACATTCTA	16	186
HAe6702Dp1	AATTCTAATACGACTCACTATAGGGAGAAGGGCA GTACACACATTCTAA	16	185
H16e6702Ap2	GATGCAAGGTCGCATATGAGACAGTTATGCACAGAGCT	16	142
H16e6702Bp2	GATGCAAGGTCGCATATGAGATATTAGAATGTGTGTAC	16	182
H16e6702Cp2	GATGCAAGGTCGCATATGAGTTAGAATGTGTGTACTGC	16	185
H16e6702Dp2	GATGCAAGGTCGCATATGAGGAATGTGTGTACTGCAAG	16	188
H16e6702Apo	ACAGTTATGCACAGAGCT	16	142
H16e6702Bpo	ATATTAGAATGTGTGTAC	16	182
H16e6702Cpo	TTAGAATGTGTGTACTGC	16	185

	Primer name	Sequence	HPV Type	nt
	H16e6702Dpo	GAATGTGTGTACTGCAAG	16	188
	HAe6701po	CTTTGCTTTTCGGGATTTATGC	16	235
	HAe6702po	TATGACTTTGCTTTTCGGGA	16	230
5	HAe6702mb1	X <sub>2</sub> -cgcatgTATGACTTTGCTTTTCGGGAcatgcg -X <sub>3</sub>	16	230
	HAe6702mb2	X <sub>2</sub> -ccagctTATGACTTTGCTTTTCGGGAagctgg -X <sub>3</sub>	16	230
	HAe6702mb3	X <sub>2</sub> -cacgcTATGACTTTGCTTTTCGGGAgcgtg -X <sub>3</sub>	16	230
10	H16e6702mb4	X <sub>2</sub> -cgatcgTATGACTTTGCTTTTCGGGAcatcg -X <sub>3</sub>	16	230
	HAe6703p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGTA	16	656
	HAe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCACAC	16	741
	HAe6703po	TGGACAAGCAGAACCGGACAGAGC	16	687
15	HAe6704p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGA	16	656
	HAe6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCA	16	741
	HAe6704po	AGCAGAACCGGACAGAGCCCATTA	16	693
	H18e6701p2	GATGCAAGGTCGCATATGAGACGATGAAATAGATGGA GTT	18	702
	H18e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACGGA CACACAAAGGACAG	18	869
20	H18e6701po	AGCCGAACCACAACGTCACA	18	748
	H18e6702p2	GATGCAAGGTCGCATATGAGGAAAACGATGAAATAGA TGGAG	18	698
	H18e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACACCA CGGACACACAAAGGACAG	18	869
	H18e6702po	GAACCACAACGTCACACAATG	18	752
25	H18e6702mb1	X <sub>2</sub> -cgcatgGAACCACAACGTCACACAATGcatgcg -X <sub>3</sub>	18	752
	H18e6702mb2	X <sub>2</sub> -ccgtcgGAACCACAACGTCACACAATGcgacgg -X <sub>3</sub>	18	752
	H18e6702mb3	X <sub>2</sub> -cggaccGAACCACAACGTCACACAATGggtccg -X <sub>3</sub>	18	752
	H18e6702mb4	X <sub>2</sub> -cgatcgGAACCACAACGTCACACAATGcgatcg -X <sub>3</sub>	18	752
	H18e6703p2	GATGCAAGGTCGCATATGAGTTCCGGTTGACCTTCTA TGT	18	651
	H18e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTCGT CTGCTGAGCTTTCT	18	817
30	H18e6704p2	GATGCAAGGTCGCATATGAGGCAAGACATAGAAATAA CCTG	18	179
	H18e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGACCCAG TGTTAGTTAGTT	18	379
	H18e6704po	TGCAAGACAGTATTGGAAC	18	207
	H31e6701p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGATG AAC	31	164

	Primer name	Sequence	HPV Type	nt
		ggg -X <sub>1</sub>		
	H33e6703mb4	X <sub>2</sub> -ccaaagcGGACAAGCACACCAGCCACAGCg	33	699
		ctttgg -X <sub>3</sub>		
30	H33e6703mb5	X <sub>2</sub> -cctgcGGACAAGCACACCAGCCACAGCgcagg -X <sub>3</sub>	33	699
	H33e6703mb6	X <sub>2</sub> -cgatcgGGACAAGCACACCAGCCACAGCcga	33	699
		tcg -X <sub>3</sub>		
	H33e6702p2	GATGCAAGGTCGCATATGAGGACCTTTGTGTCCTCAA GAA	33	431
	H33e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGGTCA GTTGGTTCAGGATA	33	618
	H33e6702po	AGAACTGCACTGTGACGTGT	33	543
	H35e6701p2	GATGCAAGGTCGCATATGAGATTACAGCGGAGTGAGG TAT	35	217
35	H35e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCTTT GCTTTTCAACTGGA	35	442
	H35e5601po	ATAGAGAAGGCCAGCCATAT	35	270
	H35e6702p2	GATGCAAGGTCGCATATGAGTCAGAGGAGGAGGAAGA TACTA	35	655
	H35e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGATTAT GCTCTCTGTGAACA	35	844
	H35e6703p2	GATGCAAGGTCGCATATGAGCCCGAGGCAACTGACCT ATA	35	610
40	H35e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCAAT GTGTGTGCTCTGTA	35	770
	H35e6702po	GACAAGCAAAAACCAGACACCTCCAA	35	692
	H35e6703po	GACAAGCAAAAACCAGACACC	35	692
	H52e6701p2	GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTGGA AGAAT	52	144
	H52e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCTCT CTTCTAATGTTT	52	358
45	H52e6701po	GTGCCTACGCTTTTTTATCTA	52	296
	H52e6702p2	GATGCAAGGTCGCATATGAGGTGCCTACGCTTTTTTAT CTA	52	296
	H52e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGGGTC TCCAACACTCTGAACA	52	507
	H52e6702po	TGCAACAAGCGATTTC	52	461
	H58e6701p2	GATGCAAGGTCGCATATGAGTCAGGCGTTGGAGACATC	58	157
50	H58e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCAAT CGTAAGCACACT	58	301
	H58e6702p2	GATGCAAGGTCGCATATGAGTCTGTGCATGAAATCGAA	58	173
	H58e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCACA CTTTACATACTG	58	291
	H58e6701po	TGAAATGCGTTGAATGCA	58	192
	H58e6702po	TTGCAGCGATCTGAGGTATATG	58	218
55	HBe6701p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAACAT	B(11)	514
	HBe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCATCT	B(11)	619

	Primer name	Sequence	HPV Type	nt
		TCTGAGCTGTCT		
	HBe6702p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAACA TGCA	B (11)	514
	HBe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCACA TCCACAGCAACAGGTCA	B (11)	693
60	HBe6701po	GTAGGGTTACATTGCTATGA	B (11)	590
	HBe6702po	GTAGGGTTACATTGCTATGAGC	B (11)	590
	HBe6703p2	GATGCAAGGTCGCATATGAGTGACCTGTTGCTGTGGA TGTGA	B (11)	693
	HBe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTACCTG AATCGTCCGCCAT	B (11)	832
	HBe6703po	ATWGTGTGTCCCATCTGC	B (11)	794
	HCe6701p2	GATGCAAGGTCGCATATGAGCATGCCATAAATGTATAGA	C (18 39 45)	295
65	HCe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACCGC AGGCACCTTATTAA	C (18 39 45)	408
	HCe6701po	AGAATTAGAGAATTAAGA	C (18 39 45)	324
	H39e6701p2	GATGCAAGGTCGCATATGAGGCAGACGACCACTACAG CAAA	39	210
	H39e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACACCG AGTCCGAGTAATA	39	344
	H39e6701po	ATAGGGACGGGGAACCACT	39	273
70	H39e6702p2	GATGCAAGGTCGCATATGAGTATTACTCGGACTCGGTGT	39	344
	H39e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTTGGG TTTCTCTTCGTGTTA	39	558
	H39e6702po	GGACCACAAAACGGGAGGAC	39	531
	H39e6703p2	GATGCAAGGTCGCATATGAGGAAATAGATGAACCCGA CCA	39	703
	H39e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAC CACGGACACACAAA	39	886
75	H39e6703po	TAGCCAGACGGGATGAACCACAGC	39	749
	HPV45p2	GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA	45	430
	HPV45p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA	45	527
	HPV45po	GTACCGAGGGCAGTGAATA	45	500
	H45e6701p2	GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA	45	430
80	H45e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA	45	527
	H45e6702p2	GATGCAAGGTCGCATATGAGGAAACCATTGAACCCAG CAGAAAA	45	428
	H45e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGCTA TACTTGTGTTTCCCTACG	45	558
	H45e6701po	GTACCGAGGGCAGTGAATA	45	500
	H45e6702po	GGACAAACGAAGATTTTCA	45	467



	Primer name	Sequence	HPV Type	nt
	H45e6703p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CCAGCAAT	45	656
	H45e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACCAC GGACACACAAAGGACAAG	45	868
	H45e6704p2	GATGCAAGGTCGCATATGAGCTGTTGACCTGTTGTGT TACGA	45	654
	H45e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCACGG ACACACAAAGGACAAG	45	868
5	H45e6705p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CGA	45	656
	H45e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGAC ACACAAAGGACAAG	45	868
	H45e6703po	GAGTCAGAGGAGGAAAACGATG	45	686
	H45e6704po	AGGAAAACGATGAAGCAGATGGAGT	45	696
	H45e6705po	ACAACCTACCAGCCCGACGAGCCGAA	45	730
10	H51e6701p2	GATGCAAGGTCGCATATGAGGGAGGAGGATGAAGTAG ATA	51	658
	H51e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCCCAT TAACATCTGCTGTA	51	807
	H51e6702p2	GATGCAAGGTCGCATATGAGAGAGGAGGAGGATGAAG TAGATA	51	655
	H51e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGGC AAACCAGGCTTAGT	51	829
	H51e6701po	GCAGGTGTTCAAGTGTAGTA	51	747
15	H51e6702po	TGGCAGTGGAAAGCAGTGGAGACA	51	771
	H56e6701p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACA AACATCT	56	519
	H56e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTTCATC CTCATCCTCATCCTCTGA	56	665
	H56e6702p2	GATGCAAGGTCGCATATGAGTGGGGTGCTGGAGACAA ACATC	56	520
	H56e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCATCCT CATCCTCATCCTCTGA	56	665
20	H56e6703p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACA AACAT	56	519
	H56e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCACAA ACTTACACTCACAACA	56	764
	H56e6701po	AAAGTACCAACGCTGCAAGACGT	56	581
	H56e6702po	AGAACTAACACCTCAAACAGAAAT	56	610
	H56e6703po	AGTACCAACGCTGCAAGACGTT	56	583
25	H56e6703po1	TTGGACAGCTCAGAGGATGAGG	56	656
	H56e6704p2	GATGCAAGGTCGCATATGAGGATTTTCCTTATGCAGT GTG	56	279
	H56e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGACATC TGTAGCACCTTATT	56	410
	H56e6704po	GACTATTCAAGTGTATGGAGC	56	348
	HPVAP01A	CAACTGAYCTMYACTGTTATGA	A (16	

Primer name	Sequence	HPV Type	nt
		31 35)	
HPVAp01Amb1	X <sub>2</sub> -cgcatgCAACTGAYCTMYACTGTTATGAcatgcg -X <sub>3</sub>	A (16 31 35)	
HPVAp01Amb2	X <sub>2</sub> -ccgtcgCAACTGAYCTMYACTGTTATGAcga cgg -X <sub>3</sub>	A (16 31 35)	
HPVAp01Amb3	X <sub>2</sub> -ccacccCAACTGAYCTMYACTGTTATGAgg gtgg -X <sub>3</sub>	A (16 31 35)	
HPVAp01Amb4	X <sub>2</sub> -cgatcgCAACTGAYCTMYACTGTTATGAcga tcg -X <sub>3</sub>	A (16 31 35)	
HPVAP04A	GAAMCAACTGACCTAYWCTGCTAT	A (33 52 58)	
HPVAP04Amb1	X <sub>2</sub> -ccaagcGAAMCAACTGACCTAYWCTGCTATgc ttgg -X <sub>3</sub>	A (33 52 58)	
HPVAP04Amb2	X <sub>2</sub> -ccaagccGAAMCAACTGACCTAYWCTGCTAT ggcttgg -X <sub>3</sub>	A (33 52 58)	
HPVAP04Amb3	X <sub>2</sub> -ccaagcgGAAMCAACTGACCTAYWCTGCTA Tcgcttgg -X <sub>3</sub>	A (33 52 58)	
HPVAP04Amb4	X <sub>2</sub> -ccagcgGAAMCAACTGACCTAYWCTGCTATcg ctgg -X <sub>3</sub>	A (33 52 58)	
HPVAP04Amb5	X <sub>2</sub> -cgatcgGAAMCAACTGACCTAYWCTGCTATcg atcg -X <sub>3</sub>	A (33 52 58)	
HPVCPO4	AAGACATTATTCAGACTC	C (18 45 39)	
HPVCPO4Amb1	X <sub>2</sub> -ccaagcAAGACATTATTCAGACTCgcttgg -X <sub>3</sub>	C (18 45 39)	
HPVCPO4Amb2	X <sub>2</sub> -cgcatgAAGACATTATTCAGACTCcatgcg -X <sub>3</sub>	C (18 45 39)	
HPVCPO4Amb3	X <sub>2</sub> -cccagcAAGACATTATTCAGACTCgctggg -X <sub>3</sub>	C (18 45 39)	
HPVCPO4Amb4	X <sub>2</sub> -cgatcgAAGACATTATTCAGACTCcgatcg -X <sub>3</sub>	C (18 45 39)	

45

Pairs of P1 and P2 primers having the same prefix (e.g. HAe6701p1 and HAe6701p2) are intended to be used in combination. However, other combinations may also be used, as summarised below for HPV types 16, 18, 31, 33 and 45.

50

Suitable primer-pairs for amplification of HPV 16 E6 mRNA are as follows:

- 52 -

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6701p1 or HAe6702p1 (both nt 368).

5 HAe6701p2 or HAe6702p2 (both nt 116) with HPV16p1 (nt 258).

10 H16e6702Ap2 (nt 142), H16e6702Bp2 (nt 182),  
H16e6702Cp2 (nt 185) or H16e6702Dp2 (nt 188) with  
HAe6701p1 or HAe6702p1 (both nt 368).

15 HAe6701p2 or HAe6702p2 (both nt 116) with HAe6702Ap1  
(nt 208), HAe6702Bp1 (nt 191), HAe6702Cp1 (nt 186) or  
HAe6702Dp1 (185). These combinations are suitable for  
amplification of all E6 splice variants.

20 HAe6703p2 or HAe6704p2 (both nt 656) with HAe6703p1 or  
HAe6704p1 (both nt 741). These combinations are  
suitable for amplification of all transcripts  
containing the E7 coding region (at least up to nt  
741).

The following primer-pairs are preferred for  
amplification of HPV 18 E6 mRNA:

25 H18e6701p2 (nt 702) or H18e6702p2 (nt 698) with  
H18e6701p1 or H18e6702p1 (both nt 869).

H18e6703p2 (nt 651) with H18e6703p1 (nt 817).

30 H18e6704p2 (nt 179) with H18e6704p1 (nt 379).

The following primer-pairs are preferred for  
amplification of HPV 31 E6 mRNA:

- 53 -

H31e6701p2 or H31e6702p2 (both nt 164) with H31e6701p1 or H31e6702p1 (both nt 423).

5 H31e6703p2 (nt 617), H31e6704p2 (nt 619) or H31e6705p2 (nt 617) with H31e6703p1 (nt 766), H31e6704p1 (766) or H31e6705p1 (nt 809).

The following primer-pairs are preferred for amplification of HPV 33 E6 mRNA:

10

H33e6701p2 (nt 618) or H33e6703p2 (nt 620) with H33e6701p1 (nt 763) or H33e6703p1 (nt 807).

H33e6702p2 (nt 431) with H33e6702p1 (nt 618).

15

The following primer pair is preferred for amplification of HPV 45:

HPV45p2 (nt 430) with HPV45p1 (nt 527)

20

Table 4-E6 PCR primers

	Primer name	Sequence	HPV type	nt
25	HAe6701PCR2	CCACAGGAGCGACCCAGAAAGTTA	16	116
	HAe6701PCR1	ACGGTTTGTGTATTGCTGTTC	16	368
	HAe6702PCR2	CCACAGGAGCGACCCAGAAA	16	116
	HAe6702PCR1	GGTTTGTGTATTGCTGTTC	16	368
	HAe6703PCR2	CAGAGGAGGAGGATGAAATAGTA	16	656
	HAe6703PCR1	GCACAACCGAAGCGTAGAGTCACAC	16	741
30	HAe6704PCR2	CAGAGGAGGAGGATGAAATAGA	16	656
	HAe6704PCR1	GCACAACCGAAGCGTAGAGTCA	16	741
	H18e6701PCR2	ACGATGAAATAGATGGAGTT	18	702
	H18e6701PCR1	CACGGACACACAAAGGACAG	18	869
	H18e6702PCR2	GAAAACGATGAAATAGATGGAG	18	698
35	H18e6702PCR1	ACACCACGGACACACAAAGGACAG	18	869
	H18e6703PCR2	TTCCGGTTGACCTTCTATGT	18	651
	H18e6703PCR1	GGTCGTCTGCTGAGCTTCT	18	817
	H18e6704PCR2	GCAAGACATAGAAATAACCTG	18	179

	Primer name	Sequence	HPV type	nt
	H18e6704PCR1	ACCCAGTGTAGTTAGTT	18	379
	H31e6701PCR2	GGAAATACCCTACGATGAAC	31	164
	H31e6701PCR1	GGACACAACGGTCTTTGACA	31	423
	H31e6702PCR2	GGAAATACCCTACGATGAAC	31	164
5	H31e6702PCR1	CTGGACACAACGGTCTTTGACA	31	423
	H31e6703PCR2	ACTGACCTCCACTGTTATGA	31	617
	H31e6703PCR1	TATCTACTTGTGTGCTCTGT	31	766
	H31e6704PCR2	TGACCTCCACTGTTATGAGCAATT	31	619
	H31e6704PCR1	TGCGAATATCTACTTGTGTGCTCT GT	31	766
10	H31e6705PCR2	ACTGACCTCCACTGTTAT	31	617
	H31e6705PCR1	CACGATTCCAAATGAGCCCAT	31	809
	H33e6701PCR2	TATCCTGAACCAACTGACCTAT	33	618
	H33e6701PCR1	TTGACACATAAACGAACTG	33	763
	H33e6703PCR2	TCCTGAACCAACTGACCTAT	33	620
15	H33e6703PCR1	CCCATAAGTAGTGTGCTGTAT	33	807
	H33e6702PCR2	GACCTTTGTGTCTCTCAAGAA	33	431
	H33e6702PCR1	AGGTCAGTTGGTTCAGGATA	33	618
	H35e6701PCR2	ATTACAGCGGAGTGAGGTAT	35	217
	H35e6701PCR1	GTCTTTGCTTTTCAACTGGA	35	442
20	H35e6702PCR2	TCAGAGGAGGAGGAAGATACTA	35	655
	H35e6702PCR1	GATTATGCTCTCTGTGAACA	35	844
	H35e6703PCR2	CCCGAGGCAACTGACCTATA	35	610
	H35e6703PCR1	GTCAATGTGTGTGCTCTGTA	35	770
	H52e6701PCR2	TTGTGTGAGGTGCTGGAAGAAT	52	144
25	H52e6701PCR1	CCCTCTCTTCTAATGTTT	52	358
	H52e6702PCR2	GTGCCTACGCTTTTTATCTA	52	296
	H52e6702PCR1	GGGGTCTCCAACACTCTGAACA	52	507
	H58e6701PCR2	TCAGGCGTTGGAGACATC	58	157
	H58e6701PCR1	AGCAATCGTAAGCACACT	58	301
30	H58e6702PCR2	TCTGTGCATGAAATCGAA	58	173
	H58e6702PCR1	AGCACACTTTACATACTG	58	291
	HBe6701PCR2	TACACTGCTGGACAACAT	B(11)	514
	HBe6701PCR1	TCATCTTCTGAGCTGTCT	B(11)	619
	HBe6702PCR2	TACACTGCTGGACAACATGCA	B(11)	514
35	HBe6702PCR1	GTCACATCCACAGCAACAGGTCA	B(11)	693
	HBe6703PCR2	TGACCTGTTGCTGTGGATGTGA	B(11)	693
	HBe6703PCR1	TACCTGAATCGTCCGCCAT	B(11)	832
	HCe6701PCR2	CATGCCATAAATGTATAGA	C (18 39 45)	295
	HCe6701PCR1	CACCGCAGGCACCTTATTAA	C (18 39 45)	408
40	H39e6701PCR2	GCAGACGACCACTACAGCAAA	39	210
	H39e6701PCR1	ACACCGAGTCCGAGTAATA	39	344
	H39e6702PCR2	TATTACTCGGACTCGGTGT	39	344
	H39e6702PCR1	CTTGGGTTTCTCTTCGTGTTA	39	558
	H39e6703PCR2	GAAATAGATGAACCCGACCA	39	703
45	H39e6703PCR1	GCACACCACGGACACACAAA	39	886
	H45e6701PCR2	AACCATTGAACCCAGCAGAAA	45	430
	H45e6701PCR1	TCTTTCTTGCCGTGCCTGGTCA	45	527
	H45e6702PCR2	GAAACCATTGAACCCAGCAGAAAA	45	428
	H45e6702PCR1	TTGCTATACTTGTGTTTCCCTACG	45	558

- 55 -

Primer name	Sequence	HPV type	nt
H45e6703PCR2	GTTGACCTGTTGTGTTACCAGCAAT	45	656
H45e6703PCR1	CACCACGGACACACAAAGGACAAG	45	868
H45e6704PCR2	CTGTTGACCTGTTGTGTTACGA	45	654
H45e6704PCR1	CCACGGACACACAAAGGACAAG	45	868
H45e6705PCR2	GTTGACCTGTTGTGTTACGA	45	656
H45e6705PCR1	ACGGACACACAAAGGACAAG	45	868
H51e6701PCR2	GGAGGAGGATGAAGTAGATA	51	658
H51e6701PCR1	GCCCATTAACATCTGCTGTA	51	807
H51e6702PCR2	AGAGGAGGAGGATGAAGTAGATA	51	655
H51e6702PCR1	ACGGGCAAACCAGGCTTAGT	51	829
H56e6701PCR2	TTGGGGTGCTGGAGACAAACATCT	56	519
H56e6701PCR1	TTCATCCTCATCCTCATCCTCTGA	56	665
H56e6702PCR2	TGGGGTGCTGGAGACAAACATC	56	520
H56e6702PCR1	CATCCTCATCCTCATCCTCTGA	56	665
H56e6703PCR2	TTGGGGTGCTGGAGACAAACAT	56	519
H56e6703PCR1	CCACAAACTTACACTCACAACA	56	764
H56e6704PCR2	GATTTTCCTTATGCAGTGTG	56	279
H56e6704PCR1	GACATCTGTAGCACCTTATT	56	410

Preferred PCR primer-pairs for HPV types 16, 18, 31 and 33 are analogous to the NASBA primer-pairs.

Table 5-Preferred L1 NASBA primers and probes

Primer name	Sequence
Onc2A2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGTAA 3'
Onc2A1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCTCCCCATGTC 3'
Onc2PoA	5' TTGTTACTGTTGTTGATACTAC 3'
Onc2B2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGSRHAA 3'
Onc2B1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCTCMMCATGDC 3'
Onc2PoB	5' TTGTTACTGTTGTTGATACYAC 3'
Onc2PoC	5' TTGTTACTGTTGTTGATACCAC 3'
Onc2C2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGSIIAA 3'
Onc2D2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGIIHAA 3'
Onc2E2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGIRIAA 3'
Onc2F2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGTAA 3'

- 56 -

5	Onc2G2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGAAA 3'
	Onc2H2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGCATAA 3'
	Onc2I2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGGGCAA 3'
	Onc2J2	5' GATGCAAGGTCGCATATGAGAATGGCATTGTTGGCACAA 3'
	Onc2K1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMICATGIC 3'
	Onc2L1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCAACATGIC 3'
	Onc2M1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGTC 3'
	Onc2N1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGGC 3'
	Onc2O1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGAC 3'
	Onc2P1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGCC 3'

Table 6-Preferred L1 PCR primers

15	Primer name	Sequence
	Onc2A1-PCR	5' AATGGCATTGTTGGGGTAA 3'
	Onc2A2-PCR	5' TCATATTCCTCCCATGTC 3'
	Onc2B1-PCR	5' AATGGCATTGTTGGSRHAA 3'
	Onc2B2-PCR	5' TCATATTCCTCMMCATGDC 3'
	Onc2C1-PCR	5' AATGGCATTGTTGGSIIAA 3'
	Onc2D1-PCR	5' AATGGCATTGTTGGIIHAA 3'
	Onc2E1-PCR	5' AATGGCATTGTTGGIRIAA 3'
	Onc2F1-PCR	5' AATGGCATTGTTGGGGTAA 3'
	Onc2G1-PCR	5' AATGGCATTGTTGGGGAAA 3'
25	Onc2H1-PCR	5' AATGGCATTGTTGGCATAA 3'
	Onc2I1-PCR	5' AATGGCATTGTTGGGGCAA 3'
	Onc2J1-PCR	5' AATGGCATTGTTGGCACAA 3'
	Onc2K2-PCR	5' TCATATTCCTCMICATGIC 3'
	Onc2L2-PCR	5' TCATATTCCTCAACATGIC 3'
30	Onc2M2-PCR	5' TCATATTCCTCIICATGTC 3'
	Onc2N2-PCR	5' TCATATTCCTCIICATGGC 3'
	Onc2O2-PCR	5' TCATATTCCTCIICATGAC 3'
	Onc2P2-PCR	5' TCATATTCCTCIICATGCC 3'

- 57 -

The HPV-specific sequences in SEQ ID NOs:149 and 150 (primers Onc2A2/Onc2A1-PCR and Onc2A1/Onc2A2-PCR) are identical to fragments of the HPV type 16 genomic sequence from position 6596-6615 (SEQ ID NO:149; 5 Onc2A2/Onc2A1-PCR), and from position 6729 to 6747 (SEQ ID NO:150; Onc2A1/Onc2A2-PCR).

The HPV-specific sequences SEQ ID NOs:152 and 153 (Onc2B2/Onc2B1-PCR and Onc2B1/Onc2B2-PCR) are variants 10 of the above sequences, respectively, including several degenerate bases. Representations of the sequences of degenerate oligonucleotide molecules provided herein use the standard IUB code for mixed base sites: N=G,A,T,C; V=G,A,C; B=G,T,C; H=A,T,C; 15 D=G,A,T; K=G,T; S=G,C; W=A,T; M=A,C; Y=C,T; R=A,G.

It is also possible to use variants of the HPV-specific sequences SEQ ID NO:152 (Onc2B2/Onc2B1-PCR) and SEQ ID NO:153 (Onc2B1/Onc2B2-PCR) wherein any two 20 of nucleotides "SRH" towards the 3' end of the sequence are replaced with inosine (I), as follows:

5' AATGGCATTGTGTTGGIIHAA 3'  
 5' AATGGCATTGTGTTGGSIIAA 3'  
 25 5' AATGGCATTGTGTTGGIRIAA 3'

The HPV-specific sequences SEQ ID NOs: 156-163 (present in primers Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2, Onc2J2, Onc2C1-PCR, Onc2D1- 30 PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR) are variants based on the HPV-specific sequence SEQ ID NO:152 (Onc2B2/Onc2B1-PCR), whereas the HPV-specific sequences SEQ ID NOs: 164-169 (present in primers Onc2K1, Onc2L1, Onc2M1, 35 Onc2N1, Onc2O1, Onc2P1, Onc2K2-PCR, Onc2L2-PCR,



- 58 -

Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR are variants based on the HPV-specific sequence SEQ ID NO:153 (Onc2B1/Onc2B2-PCR). These variants include degenerate bases and also inosine (I) residues. This sequence variation enables oligonucleotides incorporating the variant sequences to bind to multiple HPV types. Inosine bases do not interfere with hybridization and so may be included at sites of variation between HPV types in order to construct a "consensus" primer able to bind to multiple HPV types.

Any one or more of primers Onc2A2, Onc2B2, Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2 and Onc2J2, may be used in combination with any one or more of primers Onc2A1, Onc2B1, Onc2K1, Onc2L1, Onc2M1, Onc2N1, Onc2O1 and Onc2P1, for NASBA amplification of HPV L1 mRNA.

Any one or more of primers Onc2A1-PCR, Onc2B1-PCR, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR, may be used in combination with any one or more of primers Onc2A2-PCR, Onc2B2-PCR, Onc2K2-PCR, Onc2L2-PCR, Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR for PCR amplification of HPV L1 mRNA.

The invention will be further understood with reference to the following experimental examples and figures in which:

Figure 1A shows the results of a single reaction real-time NASBA assay using a FAM molecular beacon for HPV 16 on a patient sample while figure 1B shows multiplexed real-time NASBA assay using the FAM molecular beacon of Figure 1A and a molecular beacon labeled with Texas red for UIA,

- 59 -

Figure 2A shows single reaction real-time NASBA with FAM molecular beacon for HPV 18 on a patient sample while figure 2B shows a multiplexed version with a Texas red labeled molecular beacon for HPV 18 and a FAM labeled beacon for HPV 33,

Figure 3A shows single reaction real-time NASBA with HPV 31 FAM labeled molecular beacon while figure 3B shows multiplexed version including HPV 45 Texas red labeled molecular beacon,

Figure 4A shows single reaction real-time NASBA with HPV 33 FAM labeled molecular beacon while figure 4B is a multiplexed version including a Texas red labeled HPV 18 molecular beacon,

Figure 5A shows single reaction real-time NASBA with HPV45 FAM labeled molecular beacon while figure 5B shows the multiplexed version including HPV 45 Texas red labeled molecular beacon and a FAM labeled HPV 31 molecular beacon, and

Figure 6 shows HPV detected by PreTect HPV-Proofer and PCR compared to cytology or histology.

#### Example 1-Detection of HPV mRNA by NASBA-based nucleic acid amplification and real-time detection

##### Collection and preparation of clinical samples

Pap smears and HPV samples were collected from 5970 women in the cervical screening program in Oslo, Norway. Samples intended for RNA/DNA extraction were treated as follows:

Cervical samples were collected from each women attending the cervical screening program using a cytobrush(Rovers Medical Devices, The Netherlands). The cytobrush was then immersed in 9 ml lysis buffer (5M Guanidine thiocyanate). Since RNA is best

- 60 -

protected in the 5M guanidine thiocyanate at -70°C only 1 ml of the total volume of sample was used for each extraction round. The samples in lysis buffer were stored at -20°C for no more than one week, then  
5 at -70°C until isolation of DNA / RNA.

RNA and DNA were automatically isolated from 5300 women in the first round of extraction, using 1ml from the total sample of 9ml in lysis buffer. RNA and DNA  
10 were extracted according to the "Booms" isolation method from Organon Teknika (Organon Teknika B.V., Boselind 15, P.O. Box 84, 5280 AB Baxtel, The Netherlands; now Biomérieux, 69280 Marcy l'Etoile, France) using the Nuclisens™ extractor following the  
15 protocol for automated extraction.

#### Cell lines

DNA and RNA from HeLa (HPV 18), SiHa (HPV 16) and CaSki (HPV 16) cell lines were used as positive  
20 controls for the PCR and NASBA reactions. These cells were also used as sample material in the sensitivity study (Example 2). SiHa cells have integrated 1 - 2 copies of HPV 16 per cell, whilst CaSki cells have between 60-600 copies of HPV 16, both integrated and  
25 in the episomal state. HeLa cells have approximately 10-50 copies of HPV 18 per cell.

#### HPV detection and typing by PCR

Isolated DNA from cervical scrapes was subjected  
30 to PCR using the consensus GP5+/6+ primers (EP-B-0 517 704). The PCR was carried out in 50 µl reaction volume containing 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01 % Tween 20™, 200 mM each of dNTP, 1.5 mM MgCl<sub>2</sub>, 1 U recombinant Taq DNA Polymerase (MBI  
35 Fermentas), 3 µl DNA sample and 50 pmol of each GP5+

- 61 -

and GP6+ primers. A 2 minutes denaturation step at 94°C was followed by 40 cycles of amplification with a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 1 minutes,  
5 a primer annealing step at 40°C for 2 minutes and a chain elongation step at 72°C for 1.5 minutes. The final elongation step was prolonged by 4 minutes to ensure a complete extension of the amplified DNA.

10 The GP5+/6+ positive samples were subjected to HPV type 16, 31 and 33 PCR protocols as follows:  
HPV 16, 31 and 33: The PCR was carried out in 50 µl containing 75 mM Tris-HCl (pH 8.8 at 25°C), 200 mM each of dNTP, 1.5 mM MgCl<sub>2</sub>, 2.5 U recombinant Taq DNA  
15 Polymerase (MBI Fermentas), 3 µl DNA sample and 25 pmol of each primers. A 2 minutes denaturation step at 94°C was followed by 35 cycles of amplification with a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at  
20 30 sec, a primer annealing step at 57°C for 30 sec and a chain elongation step at 72°C for 1 minutes. The final elongation step was prolonged by 10 minutes to ensure a complete extension of the amplified DNA. The protocol for HPV 33 had a primer annealing step at  
25 52°C. HPV 18 protocol: Primers were designed to identify HPV type 18. The PCR was carried out in 50 µl containing 75 mM Tris-HCl (pH 8,8 at 25°C), 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01 % Tween 20, 200 mM each of dNTP, 2.0 mM MgCl<sub>2</sub>, 2.5 U recombinant Taq DNA Polymerase (MBI  
30 Fermentas), 3 µl DNA sample and 25 pmol of each primer. A 2 minutes denaturation step at 94°C was followed by 35 cycles of amplification in a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 30 sec, a primer  
35 annealing step at 57°C for 30 sec and a chain elongation step at 72°C for 1 minutes. The final

- 62 -

elongation step was prolonged by 10 minutes to ensure a complete extension of the amplified DNA.

A primer set directed against the human  $\beta$ -globin gene was used as a control of the DNA quality (Operating procedure, University Hospital Vrije Universiteit, Amsterdam, The Netherlands). The PCR was carried out in 50  $\mu$ l containing 75 mM Tris-HCl (pH 8.8 at 25°C), 200 mM each of dNTP, 1,5 mM  $MgCl_2$ , 1 U Recombinant Taq DNA Polymerase (MBI Fermentas), 3  $\mu$ l DNA sample and 25 pmol of each primer. A 2 minutes denaturation step at 94°C was followed by 35 cycles of amplification with a PCR processor (Primus 96, HPL block, MWG, Germany). Each cycle included a denaturation step at 94°C for 1 minute, a primer annealing step at 55°C for 1 ½ minutes and a chain elongation step at 72°C for 2 minutes. The final elongation step was prolonged by 4 minutes to ensure a complete extension of the amplified DNA. HeLa was used as positive controls for HPV 18, while SiHa or CaSki were used as positive control for HPV 16. Water was used as negative control.

#### Primers used for HPV PCR:

Type	Primer	Primer sequence	Position	Length (bp)
25	HPV16 Pr1	5' TCA AAA GCC ACT GTG TCC TGA 3'	421 - 440	119
	Pr2	5' CGT GTT CTT GAT GAT CTG CAA 3'	521 - 540	
	HPV18 Pr1	(5' TTC CGG TTG ACC TTC TAT GT 3')	651 - 670	186
	Pr2	(5' GGT CGT CTG CTG AGC TTT CT 3')	817 - 836	
	HPV31 Pr1	5' CTA CAG TAA GCA TTG TGC TAT GC 3'	3835 - 3875	153
	Pr2	5' ACG TAA TGG AGA GGT TGC AAT AAC CC 3'	3963 - 3988	
30	HPV33 Pr1	5' AAC GCC ATG AGA GGA CAC AAG 3'	567 - 587	211
	Pr2	5' ACA CAT AAA CGA ACT GTG TGT 3'	758 - 778	
	Gp+ Gp5+	5' TTT GTT ACT GTG GTA GAT ACT AC 3'	6624 - 6649	150
	Gp6+	5' GAA AAA TAA ACT GTA AAT CAT ATT C	6719 - 6746	
35	BGPCO3 Pr1	5' ACA CAA CTG TGT TCA CTA GC		
	BGPCO5 Pr2	5' GAA ACC CAA GAG TCT TCT CT		

Visualization of the PCR products was done on a DNA 500 chip (Agilent Technologies, USA) according to their manual. The DNA chip uses micro scale gel electrophoresis with an optimal detection limit of 0.5  
 5 -50 ng/ml. The results were interpreted using the Bioanalyzer 2100 software (Agilent Technologies, USA).

The following table confirms primers used for HPV PCR in patient samples and indicates additional PCR  
 10 primers useful for HPV 35, 39, 45, 51, 52, 58 and HPV 6/11.

PCR primers for detection of HPV.

	Type	Primer	Primer sequence	Position	Length (bp)
15	HPV 6/11	Pr1	5' TAC ACT GCT GGA CAA CAT 3'	514 - 531	123
		Pr2	5' TCA TCT TCT GAG CTG TCT 3'	619 - 636	
	HPV16	Pr1	5' TCA AAA GCC ACT GTG TCC TGA 3'	421 - 441	120
		Pr2	5' CGT GTT CTT GAT GAT CTG CAA 3'	520 - 540	
	HPV18	Pr1	5' TTC CGG TTG ACC TTC TAT GT 3'	651 - 670	186
		Pr2	5' GGT CGT CTG CTG AGC TTT CT 3'	817 - 836	
	HPV31	Pr1	5' CTA CAG TAA GCA TTG TGC TAT GC 3'	3835 - 3857	155
20		Pr2	5' ACG TAA TGG AGA GGT TGC AAT AAC CC 3'	3964 - 3989	
	HPV33	Pr1	5' AAC GCC ATG AGA GGA CAC AAG 3'	567 - 587	212
		Pr2	5' ACA CAT AAA CGA ACT GTG GTG 3'	758 - 778	
	HPV 35	Pr1	5' CCC GAG GCA ACT GAC CTA TA 3'	610-629	231
		Pr2	5' GGG GCA CAC TAT TCC AA ATG 3'	821-840	
	HPV 39	Pr1	5' GCA GAC GAC CAC TAC AGC AAA 3'	210 - 230	153
25		Pr2	5' ACA CCG AGT CCG AGT AAT A 3'	344 - 362	
	HPV 45	Pr1	5' GAA ACC ATT GAA CCC AGC AGA AAA 3'	428 - 451	154
		Pr2	5' TTG CTA TAC TTG TGT TTC CCT ACG 3'	558 - 581	
	HPV 51	Pr1	5' GGA GGA GGA TGA AGT AGA TA 3'	658 - 677	169
		Pr2	5' GCC CAT TAA CAT CTG CTG TA 3'	807 - 826	
	HPV 52	Pr1	5' GTG CCT ACG CTT TTT ATC TA 3'	296 - 315	233
		Pr2	5' GGG GTC TCC AAC ACT CTG AAC A 3'	507 - 528	
	HPV 58	Pr1	5' TCA GGC GTT GGA GAC ATC 3'	157 - 174	162
		Pr2	5' AGC AAT CGT AAG CAC ACT 3'	301 - 318	
	Gp+	Gp5+	5' TTT GTT ACT GTG GTA GAT ACT AC 3'		150
30		Gp6+	5' GAA AAA TAA ACT GTA AAT CAT ATT C		
	BGPCO3	Pr1	5' ACA CAA CTG TGT TCA CTA GC		
	BGPCO5	Pr2	5' GAA ACC CAA GAG TCT TCT CT		

- 64 -

NASBA RNA amplification

Precautions for avoiding contamination:

1. Perform nucleic acid release, isolation and amplification/detection in separate laboratory areas.
- 5 2. Store and prepare reagents for nucleic acid release, isolation and amplification/detection at the laboratory areas where nucleic acid release, isolation and amplification/detection are to be performed, respectively.
- 10 3. Keep all tubes and vials closed when not in use.
4. Pipettes and other equipment that have been used in one laboratory area must not be used in the other areas.
5. Use a fresh pipette or pipette tip for each
- 15 pipetting action.
6. Use pipettes with aerosol resistant tips for fluids possibly containing nucleic acid. Pipetting of solutions must always be performed out of or into an isolated tube that is opened and closed exclusively
- 20 for this action. All other tubes and vials should be kept closed and separated from the one handled.
7. Use disposable gloves when working with clinical material possibly containing target-RNA or amplified material. If possible, change gloves after each
- 25 pipetting step in the test procedure, especially after contact with possibly contaminated material.
8. Collect used disposable material in a container. Close and remove container after each test run.
9. Soak tube racks used during nucleic acid isolation
- 30 or amplification/detection in a detergent (e.g. Merck Extran MA01 alkaline) for at least one hour after each test run.

The following procedure was carried out using reagents  
35 from the Nuclisens™ Basic Kit, supplied by Organon

- 65 -

Teknika.

Procedure for n=10 samples:-

- 5     1. Prepare enzyme solution.  
Add 55  $\mu$ l of enzyme diluent (from Nuclisens™ Basic Kit; contains sorbitol in aqueous solution) to each of 3 lyophilized enzyme spheres (from Nuclisens™ Basic Kit; contains AMV-RT, RNase H, T7 RNA polymerase and  
10     BSA). Leave this enzyme solution at least for 20 minutes at room temperature. Gather the enzyme solutions in one tube, mix well by flicking the tube with your finger, spin down briefly and use within 1 hour. Final concentrations in the enzyme mix are 375  
15     mM sorbitol, 2.5  $\mu$ g BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase.
  
2. Prepare reagent sphere/KCl solution.  
For 10 samples: add 80  $\mu$ l reagent sphere diluent (from  
20     Nuclisens™ Basic Kit; contains Tris/HCl (pH 8.5), 45% DMSO) to the lyophilized reagent sphere (from Nuclisens™ Basic Kit; contains nucleotides, dithiotreitol and MgCl<sub>2</sub>) and immediately vortex well. Do this with 3 reagent spheres and mix the solutions  
25     in one tube.
  
- Add 3  $\mu$ l NASBA water (from Nuclisens™ Basic Kit) to the reconstituted reagent sphere solution and mix well.  
30
  
- Add 56  $\mu$ l of KCl stock solution (from Nuclisens™ Basic Kit) and mix well. Use of this KCl/water mixture will result in NASBA reactions with a final KCl concentration of 70 mM. Final concentrations in the  
35     reagent/KCl solution are 1 mM of each dNTP, 2 mM of



- 66 -

ATP, UTP and CTP, 1.5 mM GTP, and 0.5 mM ITP, 0.5 mM dithiotreitol, 70 mM KCl, 12 mM MgCl<sub>2</sub>, 40 mM Tris-HCl (pH 8.5).

- 5     3. Prepare primer/probe solution containing target-specific primers and molecular beacon probe.
- For each target reaction transfer 91  $\mu$ l of the reagent sphere/KCl solution (prepared in step 2) into a fresh tube. Add 25  $\mu$ l of primers/molecular beacon probe
- 10     solution (to give final concentration of ~0.1-0.5  $\mu$ M each of the sense and antisense primers and ~ 15-70 pmol molecular beacon probe per reaction). Mix well by vortexing. Do not centrifuge.
- 15     In case less than 10 target RNA amplifications are being performed refer to the table below for the appropriate amounts of reagent sphere solution, KCl/water solution and primers to be used. Primer solutions should be used within 30 minutes after
- 20     preparation.

Reactions (n)	Reagent sphere solution ( $\mu$ l)	KCl/water ( $\mu$ l)	Primer mix ( $\mu$ l)
10	80	30	10
9	72	27	9
25     8	64	24	8
7	56	21	7
6	48	18	6
5	40	15	5
4	32	12	4
30     3	24	9	3
2	16	6	2
1	8	3	1

#### 4. Addition of samples

- 35     For each target RNA reaction:
- In a 96 well microtiter plate pipette 10  $\mu$ l of the primer/probe solution (prepared in step 3) into each

- 67 -

of 10 wells. Add 5  $\mu$ l nucleic acid extract to each well. Incubate the microtiter plate for 4 minutes at  $65 \pm 1$  °C. Cool to at  $41 \pm 0.5$  °C for 4 minutes.

Then to each well add 5  $\mu$ l enzyme solution.

- 5 Immediately place the microtiter plate in a fluorescent detection instrument (e.g. NucliSens™ EasyQ Analyzer) and start the amplification.

#### Results from clinical study

- 10 Table 7 shows the distribution of real-time NASBA HPV positive (L1 and/or E6 expression) and PCR HPV positive cases related to cytology results. PCR amplification was carried out as described by Karlsen et al., J Clin Microbiol. 34: 2095-2100, 1996. The
- 15 figures for expected histology are based on average results from similar study on CIN III lesions (Clavel et al., Br J Cancer, 84: 1616-1623, 2001). The results from several example cases are listed in Table 8.

20

Table 7:

	Normal	Benign	Condyloma	CIN III
<b>Cytology</b>	4474	66	16	15
<b>PCR</b>	9.0%	44.6%	87.5%	73.3%
25 <b>Real-time NASBA</b>	1%	24.6%	37.5%	73.3%
<b>Expected Histology</b>	0.2%	5-15%	15-20%	71%

30

Table 8:

Internal No.	Cytology	PCR	L1 NASBA	E6 NASBA
84	Neg	Neg	Neg	31

- 68 -

	289	Neg	31	Pos	31
	926	Neg	Neg	Pos	16
	743	Benign	Neg	Neg	33
	1512	Benign	16	Pos	16
5	3437	Benign	Neg	Neg	18
	3696	Benign	16	Pos	Neg
	2043	Condyloma	16, 51	Pos	16
	3873	Condyloma	16, 51	Pos	16
	3634	CIN II	33	Neg	33
10	4276	CIN III	Neg	Neg	18
	4767	CIN III	18	Neg	18
	1482	CIN III	Neg	Pos	16
	5217	CIN III	31	Neg	31
15	4696	CIN III	Neg	Neg	Neg

Example 2-Sensitivity of real-time NASBA on control cell lines

20 Cervical cancer cell lines, CaSki, SiHa and HeLa were diluted in lysis buffer either before automated extraction of nucleic acids using the Boom's extraction method from Organon Teknika/bioMerieux (parallels 1 and 3), or after nucleic acid extraction

25 (parallel 2). Real-time NASBA was performed using molecular beacons probes labelled with Texas red (16, L1 and 18) or FAM (U1A, 33 and 31) following the protocol described above.

30

Table 9:

		CaSki						CaSki						HeLa					
Primer sets and probes		16 E6	U1	16 E6	U 1	16 E6	U1	L1	33 E6	L1	33 E6	L1	33 E6	18 E6	31 E6	18 E6	31 E6	18 E6	31 E6
5	Parallels	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
	Number of Cells																		
10	100 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
	10 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
	1 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
	100	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
	10	+	+	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-
15	1	-	-	+	-	+	+	-	-	-	-	-	-	+	-	+	-	+	-
	10 <sup>-1</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Thus, it is possible to detect HPV E6 mRNA in less than 1 cell using real-time NASBA.

20 Real-time NASBA was tested both as a multiplex assay and as single reactions. The results from the following sensitivity study are based on parallel runs of CaSki, SiHa and HeLa cell lines, and on three parallel runs on synthetic DNA oligos for HPV type 16, 18, 31 and 33. The definition of the detection limit is that both of the samples in the parallel are positive. The number in the brackets (x) indicates that the specified amount of cells also have been detected in some runs. Sensitivity is defined as the amount of cells necessary for detection of HPV in two parallel runs. The HPV types are determined from PCR and the specificity is based on NASBA compared to PCR.

#### Sensitivity

35 PCR: the HPV consensus PCR using Gp5+/6+ detected only down to 10<sup>4</sup> SiHa and HeLa cells, and down to 10<sup>3</sup> CaSki cells. However, the type specific PCR primer-sets were more sensitive, detecting 10<sup>3</sup> (10<sup>2</sup>) SiHa cells and 0.1 CaSki cells for HPV 16 type specific PCR primer-set, while the HPV 18 type specific PCR primer-set detected

- 70 -

10<sup>2</sup> HeLa cells.

Real-time NASBA: Real-time NASBA with primers specific for U1A, detected 10(1) SiHa and CaSki cells and 1  
5 HeLa cell in the reaction mixture. For the HPV 16 specific primers, the lower detection limit was (10) (10<sup>2</sup>, 1) SiHa cells and 10 (1) CaSki cells and for the HPV 18 specific primers the detection limit was 1 (0.1) HeLa cell. The universal L1 primers detected 10  
10 CaSki cells. HeLa cells and SiHa cells were not detected with the universal L1 primers.

Real-time multiplex NASBA with the U1A specific primers, had a lower detection limit of 10<sup>2</sup>(10) SiHa  
15 cells and 10(1) CaSki cells when combined with the HPV 16 specific primers, which had a lower detection limit for 10(1) SiHa and 10(1) CaSki cells. The L1 specific primers in combination with the HPV 33 specific primers detected 10<sup>3</sup>(102) CaSki cells. There was no  
20 competing HPV 33 sample in the reaction. For the HPV 18 specific primers, the lower detection limit was 1 (0.1) HeLa cell when combined with the HPV 31 specific primers. There was no competing HPV 31 sample in the reaction. Sensitivity of the HPV 31 and HPV 33  
25 specific primers were not tested, due to lack of cell lines harbouring these HPV types. They were tested against samples containing HPV 31 and HPV 33, but the amount of cells and the copy number of HPV 31 and HPV 33 in these cells were unknown and most probably  
30 varied in different samples.

Table 10: sensitivity of real-time NASBA compared to PCR

Primer	NASBA			PCR		
	SiHa	CaSki	HeLa	SiHa	CaSki	HeLa
GP5+/6+	-	-	-	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>3</sup>
L1	-	10 <sup>3</sup> (10 <sup>3</sup> )	-	-	-	-
U1A	10 <sup>2</sup> (10)	10 (1)	-	-	-	-
HPV 16	10 (1)	10	-	10 <sup>3</sup> (10 <sup>3</sup> )	0.1	-
HPV 18	-	-	1 (0,1)	-	-	10 <sup>3</sup>

Real-time NASBA was performed on samples from women admitted to Østfold Central Hospital for treatment of CIN in the period of 1999-2001 (see example 3). Molecular beacon probes labeled with FAM or Texas red were used together with the nucleic acid extraction and NASBA protocols described above. The results are shown in figures 1A and 1B (HPV 16 - patient sample 205), figures 2A and 2B (HPV 18 - patient sample 146), figures 3A and 3B (HPV 31 - patient sample 236), figures 4A and 4B (HPV 33 - patient sample 218) and figures 5A and 5B (HPV 45 - patient sample 343). In each case, the "A" figure is a single reaction while the "B" figure is the multiplex assay.

Specificity: Cross reactivity of Real-time NASBA. Real-time NASBA primer combinations were tested against 490 cervical samples from the Oslo study positive with PCR for HPV 6/11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 58 or HPV X to check for cross reactivity between HPV types using NASBA. All samples have been typed by consensus PCR and type specific PCR for the respective HPV types, except for HPV type 39(2), 52(1) and 58(2). These samples are added to test against PreTect HPV-Proofer. HPV X are positive for consensus Gp5+/6+ PCR but negative for HPV6/11, 16, 18, 31, 33, 35, 45 and 51 by type specific PCR. Results are shown in table 14. No cross-reactivity was shown. Sequence confirmation of a selected number

- 72 -

of cases from table 14 is shown in table 14a.

5 PCR: a total of 773 cervical samples were tested with  
PCR and the PreTect HPV-Proofer (Real time multiplex  
NASBA), and a total of 24.6% (190/773) samples were  
positive with the Gp5+/6+ consensus PCR primers. 74.1%  
(83/112) were typed to be HPV 16, 13% (15/112) HPV 18,  
17% (19/112) HPV 31 and 12% (13/112) HPV 33 including  
multiple HPV infections. A total of 103 samples had  
10 single or multiple HPV infections, and 91.3% (94/103)  
had only a single HPV infection. Double HPV infections  
occurred in 8.7% (9/103) of the samples. All samples  
were first tested with the consensus Gp5+/6+ PCR  
primers. The HPV PCR negative samples from the  
15 consensus Gp5+/6+ were then tested with  $\beta$ -globin  
control primers for a verification of intact DNA. The  
HPV PCR positive samples were not subjected to this  
DNA control. The HPV negative samples in this study  
were all positive with  $\beta$ -globin control PCR primers.  
20 Only DNA samples positive with Gp5+/6+ PCR were  
subjected to HPV type specific PCR. HPV types of  
interest were HPV 16, 18, 31 and 33.

25 Real-time multiplex NASBA: For the real-time NASBA  
reactions, the primers and probes for the U1A gene  
product were used as a performance control for intact  
RNA. Samples negative for U1A were rejected.

A total of 14.2% (110/773) of the samples were  
positive with at least one of the HPV type-specific  
30 NASBA primers including samples showing multiple HPV  
infections. From these samples 54.5% (60/110) were  
positive with HPV 16 NASBA primers, 13.6% (15/110)  
with HPV 18 primers, 21.8% (24/110) with HPV 31  
primers and 13.6% (15/110) with HPV 33 primers. A  
35 total of 45 samples were positive with the L1  
consensus primers and usually together with HPV 16

- 73 -

E6/E7 oncogene expression 82.2% (37/45). The consensus L1 was detected in 2.2% (1/45) together with either HPV 18, 31 and 33 respectively. L1 was also detected alone in 8.9% (4/45) cases, and they all were PCR  
5 positive with Gp5+/6+ primers. A total of 108 samples had single or multiple HPV infections, and 98.1% (106/108) had only a single HPV infections. Double mRNA expression occurred in 1.9% (2/108) of the samples.

10

Real-time multiplex NASBA compared to PCR: a total of 87 samples showed presence of HPV 16 DNA or RNA with HPV 16 PCR or PreTect HPV-Proofer. 64.4% (56/87) were determined to be positive for HPV 16 with both PCR and  
15 real-time NASBA. 39.1% (34/87) were only positive with PCR and 3.4% (3/87) were positive only with real-time NASBA. For HPV 18, a total of 20 samples showed presence of HPV 18 DNA or RNA with either PCR or real-time NASBA. From these 20 samples, 50% (10/20) were  
20 positive with both tests, and 35% (7/20) were only positive with PCR and 15% (3/20) were only positive with real-time NASBA. A total of 27 samples showed presence of HPV 31 DNA or RNA with either PCR or real-time NASBA. Out of these 27 samples, 59.3% (16/27)  
25 were positive with both tests and 11.1% (3/27) were positive only with the PCR test and 18.5% (5/27) were only positive with the real-time NASBA test. For HPV 33, a total of 18 samples showed presence of HPV DNA or RNA with either PCR or PreTect-HPV Proofer and  
30 55.6% (10/18) of the samples were tested positive with both tests. 16.7% (3/18) were only positive with PCR and 22.2% (4/18) were only positive with real-time NASBA.

35



Table 11: statistical distribution of HPV in samples with PCR and real-time NASBA

		PCR	%	NASBA	%
5	Total samples	773		773	
	Total positive samples	190	24.6	110	14.2
	HPV16	83	74.1	60	54.5
	HPV 18	15	13	15	13.6
10	HPV 31	19	17	24	21.8
	HPV33	13	12	15	13.6
	HPV X	78	69.6	-	-

Table 12: correspondence between PCR and real-time NASBA

20

	Total	Both tests	%	Only PCR +	% (PCR)	% (Total)	Only NASBA	% NASBA	% (total)
HPV 16	87	56	64.4	34	41.0	39.1	3	5	3.4
HPV 18	20	10	50.0	7	46.7	35.0	3	20	15.0
HPV 31	27	16	59.3	3	15.8	11.1	5	20.8	18.5
HPV 33	18	10	55.6	3	23.1	16.7	4	26.7	22.2

Table 13: Real-time NASBA results for L1

30		Total	%
	L1 (NASBA)	45	100
	L1 + HPV 16	37	82.2
	L1 alone	4	8.9
	L1 + HPV 18	1	2.2
	L1 + HPV 31	1	2.2
	L1 + HPV 33	1	2.2

Table 14: Genetic specificity of real-time multiplex NASBA compared to PCR

NASBA Primers	HPV (PCR)												Total Number
	6/11	16	18	31	33	35	39	45	51	52	58	X	
16	2	28	1	0	1	1	0	0	5	0	0	2	
18	1	1	18	0	1	0	0	0	1	0	0	1	
31	1	0	1	13	1	5	0	1	1	0	0	0	
33	1	2	0	2	12	2	0	1	2	0	0	1	
45	2	1	1	1	1	1	0	17	1	0	0	1	
Sum tested	43	71	36	32	25	23	2	23	31	1	2	201	490

Table 14a: DNA sequencing from Gp5+ PCR primers (not to be included in the article)

Int No	HPV type by PCR	HPV type by PreTect HPV-Proofer	HPV type by Sequencing (BLAST)
1272	16	16	16
152	35		35
2655	58		58
2924	33	33	33
2942	18	18	18
2987	16	16	16
3016	33	33	33
3041	35		35
3393	35		35
3873	16	16	16
4767	18	18	18
5707	18	18	18
845	X		39

### Discussion

Sensitivity of real-time NASBA was generally better than the sensitivity of PCR. The general sensitivity of real-time NASBA for all the markers were between 1 and  $10^2$  cells, which is considerable better than for the PCR reaction with a sensitivity range from  $10^2$  to  $10^4$ . As expected, the sensitivity of the specific primers and probes were better than the

- 76 -

sensitivity of the universal primers and probes.  
Real-time NASBA was just as sensitive or more  
sensitive than real-time multiplex NASBA.

5           Real-time NASBA primers and molecular beacon  
probe directed towards U1A (a human house keeping  
gene) were used as a performance control of the sample  
material in the real-time NASBA reaction to ensure  
that the RNA in the sample material was intact. A  
10       positive signal from this reaction was necessary for a  
validation of the real-time NASBA reaction.

          The sensitivity of the universal real-time NASBA  
with L1 (the major capsid protein of HPV) was much  
15       better than for the universal Gp5+/6+ PCR, also  
directed against L1, with a sensitivity of 10 cells  
compared to  $10^3$  ( $10^2$ ) CaSki cells. These two primer  
sets (PCR and NASBA) have their targets in the same  
region of the conserved L1 gene of different HPV  
20       types. The differences in sensitivity may be due to  
the fact that there is usually one copy of each gene  
per cell, while the copy number of mRNA may be several  
hundreds. The real-time NASBA L1 primers did not  
detect SiHa or HeLa cells as the Gp5+/6+ PCR primers  
25       did, indicating lack of L1 expression in these cell  
lines. Gp5+/6+ PCR primers detected  $10^4$  SiHa or HeLa  
cells. Considering the amount of HPV copies in each  
cell, it makes sense that the CaSki cells were  
detected in 1/10 the amount of cells from SiHa and  
30       HeLa since CaSki cells have 60-600 HPV copies per  
cell, both integrated and episomal, while SiHa cells  
have 1-2 HPV copies integrated per cell and HeLa cells  
have 10-50 HPV copies integrated per cell. The L1  
primer set detected only CaSki cells, with both  
35       integrated and episomal forms of HPV, and not in SiHa  
or HeLa cells, with only integrated forms of HPV. This

- 77 -

might indicate that the L1 gene is only expressed in episomal states of HPV infection, and therefore L1 may be a valuable marker for integration and persistence of HPV infection.

5

The HPV type-specific NASBA primers are directed against the full length E6/E7 transcript, which are expressed in large amount in cancer cells due to lack of E2 gene product. The real-time NASBA 16 type specific primers detected 10(1) SiHa cells and 10(1) CaSki cells compared to HPV 16 PCR primers that detected  $10^3(10^2)$  SiHa cells. The explanation for this might be the different amount of HPV copies in each cell line. The CaSki cells have both integrated and episomal forms of HPV, while SiHa has only integrated forms of HPV. This may be due to high expression of mRNA from the E6/E7 genes. For detection of CaSki cells, the detection limit for the NASBA HPV 16 primers were 10(1) CaSki cells compared to 0.1 CaSki cells for the HPV 16 PCR primers. This is peculiar, but an explanation may be that the CaSki cells contain from 60-600 copies of HPV 16 DNA, so that it is possible to detect 0.1 CaSki cells with 6-60 HPV 16 DNA copies. The lower sensitivity of real-time NASBA compared to PCR may indicate that the expression of E6/E7 in the CaSki cells is moderate/low. Degradation of the unstable mRNA may also be an explanation. The amount of HPV copies in the CaSki cells may be in the order of 60-600 times more than in the SiHa cells, which is shown by the more sensitive detection of CaSki cells.

The type specific HPV 18 PCR primers detected  $10^2$  HeLa cells. This is a magnitude of 100 better than the HPV consensus Gp5+/6+ primers and states that specific primers are generally more sensitive than consensus

35

- 78 -

primers. The sensitivity of the type specific HPV 18 NASBA primers was 1 (0,1) HeLa cells, indicating high expression of E6/E7 in HeLa cells.

5           The sensitivity of U1A NASBA primers was 10 SiHa or CaSki. The target for the U1A primer set is a human housekeeping gene that is expressed in every human cell.

10           The sensitivity of PCR and NASBA varies for different primer sets and sample material, and generally type specific primers are more sensitive than consensus primers due to base pair mismatch in consensus primer sets. The annealing temperature for  
15           the primers in the PCR reaction can be optimised, giving optimal reaction condition for the primers. In contrast to the annealing temperature in PCR, the annealing temperature for the NASBA primers must be  
20           fixed at 41°C. This lack of temperature flexibility may make the NASBA primers less sensitive and specific than the PCR primers.

          PCR amplifies double stranded DNA and the target is usually present as one copy per cell and this makes  
25           it vulnerable to the number of cells in the sample material. The target for the NASBA reaction is RNA, and mRNA may be present as multiple copies per cell, depending on the expression of the genes. By choosing a gene that is highly expressed, the mRNA copy number  
30           may be several hundred per cell and therefore easier to detect.

          dsDNA is relatively stable in the cell and the material stays intact for a long time. In contrast to  
35           dsDNA, mRNA is generally not very stable and

- 79 -

degradation of mRNA is rapid depending on the cell. There is no detected DNase or RNase activity in the lysis buffer so both dsDNA and ssRNA should be stable. Autocatalytic activity may degrade both DNA and RNA.

5 The DNA/RNA from the cervix sample should stay intact, when stored in the lysis buffer, for 24 hours at 15-30°C, 7 days at 2-8°C or at -70°C for long term storage.

10 A limitation in the real-time NASBA reaction is the concentration of the molecular beacon probes. The amount of products will exceed the concentration of the molecular beacon probes and therefore it will not be detected because a high molecular beacon probe

15 concentration will make the reaction mixture more complex and inhibit the amplification reaction. Nucleotides may also be a limitation to the final amount of the amplification product, both in the PCR and in the NASBA reaction. The final concentration of

20 the amplified product may in itself inhibit further amplification because of the amount of product and the complexity of the reaction mixture. During a NASBA reaction in the presence of molecular beacons, the probe might compete with the amplification by

25 hybridising to the template, making it unavailable for following RNA synthesis. In this way, RNA is subtracted as substrate for the reverse transcription steps and further RNA synthesis by T7 RNA polymerase. This competition is not significant with low amounts

30 of molecular beacon, and with a high amount of molecular beacon this inhibition can be overcome by a higher number of copies of input RNA.

35 The linear relationship between the amount of input RNA and the time-to-positive signal was tested in a ten-fold serial dilutions of different HPV cell

- 80 -

lines. There was a clear indication that a positive signal was dependent on the amount of input RNA and time. The multiplex reaction needed more time than the single reaction to show a positive signal. This might  
5 be due to competition in the more complex mixture in the multiplex reaction vessel and also to the fact that the multiplex reaction has a different and lower concentration of primer and probe. The relationship  
10 between amount of target RNA and time to positive signal opens up for a real-time multiplex quantitative amplification reaction with internal RNA standards in each reaction vessel.

Real-time NASBA: single vs. multiplex. Real-time NASBA  
15 was generally more sensitive than real-time multiplex NASBA. This was as expected because of competition between primers and probes in the multiplex reaction. The final concentration of primers and molecular  
20 beacon probes were optimised in the multiplex reaction so that for at least one of the primer and probe sets the concentration were lower than in the single reaction. From this it follows that with a lower  
25 concentration of primers, the less sensitive the reaction, or at least the less rapid the reaction. It will take longer time to reach the exponential stage of the amplification reaction and therefore longer  
time to detect the products. The concentration of the primers will not be a limitation to the final  
30 concentration of the product in the NASBA reaction because the double stranded DNA created from the primers will continue to serve as a template for the RNA polymerase over and over again in a loop. The  
sensitivity of multiplex real-time NASBA was the same  
for HPV 16 and HPV 18 compared to single real-time  
35 NASBA, but the sensitivity for L1 decreased drastically from a detection limit of 10(1) in the

- 81 -

single reaction to  $10^3(10^2)$  CaSki cells in the multiplex reaction. For U1A NASBA primers, the sensitivity decreased from  $10(1)$  to  $10^2(10)$  SiHa cells, while the detection limit remained the same for the CaSki cells. This decrease in detection limit may be to more complex competition of primers and molecular beacon probes in the multiplex reaction. The final concentration of primers and molecular beacon probes may not be the best and the different primers and molecular beacon probes in the multiplex reaction may interfere with each other. The U1A NASBA primers detected 1 HeLa cell. One might expect the same detection level in all the cell lines, but the sensitivity of HeLa cells were 1/10 of the detection level of SiHa and CaSki cells. These cell lines are cancer cells and they might have different impact on the cells so that the expression of U1A is different. The differences may also be due to different amount of cells in each reaction, because of counting errors during harvesting of the cells.

Real-time NASBA showed no cross reactivity between HPV 16, 18, 31 and 33 or with HPV 6/11, 35, 39, 45, 51, 52, 58 or HPV X.

The specificity of the PCR reaction may be better than the specificity of the real-time NASBA reaction because the NASBA reaction is an isothermal reaction at  $41^\circ\text{C}$  with no possibilities to change the annealing temperature of the primers. The primers are basically designed the same way as for the PCR primers. In a PCR reaction, you have the possibility to change the annealing temperature, in contrast to the NASBA reaction, and therefore choose an annealing temperature that is optimal for the two primers. This makes the annealing of the primers more specific. The



- 82 -

PCR results were visualized with gel electrophoresis. But the molecular beacon probes in the real-time NASBA reaction is an additional parameter compared to PCR and therefore may give the overall NASBA reaction a better specificity. It is also easier to find two different regions on the DNA sequence for primer annealing because there is much greater flexibility in the length of the PCR product, than for the NASBA product, which should be less than 250 bp. It is important for the specificity of the NASBA reaction to choose a unique area that is not conserved among the different HPV types. A couple of base pair mismatches may still give an amplification or hybridisation of the target.

15

Detection of CaSki (integrated and episomal state) cells with the universal L1 NASBA primers and not SiHa or HeLa (both integrated) may give an indication that integrated HPV doesn't show any L1 expression, while HPV in the episomal state may have L1 expression.

20

In summary, an identification assay has been developed for HPV type 16, 18, 31 and 33 that can accurately identify the oncogenic E6/E7 expression of these HPV types. The assay can also identify the expression of the major capsid protein, L1.

25

### Example 3-Further clinical study in 190 patients

30

#### Patients/Clinical samples

Biopsies from 190 women admitted to Østfold central-hospital for treatment of CIN in the period 1999-2001. The mean age of the 190 women included in the study was 37.4 years (range 22-74 years). Biopsies

35

- 83 -

were frozen in -80°C immediately after collection.

Cytological examination of samples

5 The routine cytological reports were used to record  
cytological findings. No attempt was made to  
re-evaluate the slides. Each one of them indicated a  
CIN II-III condition, i.e. a high grade dysplasia or  
HSIL, which was the basis for hospital admittance,  
colposcopy and biopsy.

10

Histological examination of samples

A biopsy, here termed *biopsy 1*, was taken after a  
high-grade cytology report. If it confirmed a  
high-grade lesion (CIN II or III), the patient was  
15 again admitted to hospital, this time for  
colposcopically guided conization. Before the  
conization, but after local anesthesia was applied, a  
second biopsy (*biopsy 2*) was taken from an area of  
portio where a dysplasia was most likely to be  
20 localised, judged from the gross findings. This biopsy  
(2 x 2 mm) was frozen within 2 minutes in a -80°C  
freezer.

*Biopsy 2* was split in two when frozen and half  
25 was used for DNA/RNA extraction. The other half was  
fixed in 10% buffered formaldehyde and processed for  
histopathological examination. Some lesions were not  
correctly oriented in the paraffin block and had to be  
reoriented or serial sectioned in order to show the  
30 relevant surface epithelium. Consequently, it cannot  
be guaranteed that exactly the same tissue was used  
for the extraction and for the histopathological  
evaluation. The cone specimen, finally, was evaluated  
by the local pathologist, who in all cases could  
35 confirm the presence of dysplasia. It was not always

the same grade as in the original biopsy, and, in many cases, not the same as in biopsy 2.

#### Extraction of nucleic acids

5           Nucleic acids were isolated using the automated Nuclisens Extractor as previously described (Boom et al., 1990). Each biopsy was cut in two pieces, one intended for histological examination and the other half for RNA analysis. The material intended for RNA  
10 analysis was divided into smaller pieces while kept on dry ice (-80°C) and put into 1 ml of lysisbuffer (as above) followed by 20 seconds of homogenisation using disposable pestles. 100 ml of the sample was further diluted 10 fold in lysisbuffer and 100 ml was then  
15 extracted for DNA/RNA. The extracted DNA/RNA was eluted with ~40 ml of elution buffer (Organon Teknika) and stored at -70°C.

20           All molecular beacon probes used in this study employ the fluorophore FAM (6-carboxyfluorescein) at the 5'end of the structure. This was bound to a variable stem-loop sequence coupled to the universal quencher 4-(4'dimethylaminophenylazo)benzoic acid (DABCYL) at the 3'end. The probes were delivered by  
25 Eurogentec, Belgium. Final concentration of MBs used in the reaction was 2.5 mM. For the real-time NASBA we made use of the NucliSens Basic Kit (Organon Teknika, Netherlands), intended for the development of user-defined RNA amplification assays. The NASBA  
30 amplification was carried out in a volume of 20 µl. The primer-sets and probes were directed against full-length E6/E7 mRNA for the high-risk HPV 16,18, 31, and 33. As performance control, to avoid false negative results due to degradation of nucleic acid,  
35 we used a primer set and probe directed against the human U1 small nuclear ribonucleoprotein (snRNP)

- 85 -

specific A protein (U1A mRNA) (Nelissen et al., 1991). All samples were run in duplicate on separate machines (microplate readers for measuring fluorescence and absorbance, Bio-tek FL-600 FA from MWG). mRNA  
5 isolated from CaSki/SiHa or HeLa cells served as positive controls for HPV 16 and HPV 18 transcripts, respectively. Negative controls, included for every 7 reaction, consisted of a reaction containing all reagents except mRNA.

10

#### HPV DNA analysis; Polymerase Chain Reaction

The same extracts and amounts as used in the NASBA reaction were used for PCR. The L1 consensus primers Gp5+/Gp6+ were used to detect all samples  
15 containing HPV-DNA. The PCR amplification was carried out as described above. The first DNA denaturation was done for 2 minutes at 94°C, then 40 cycles of PCR were run: denaturation 1 minute at 94°C, annealing for 2 minutes at 40°C, extension for 1.5 minutes at 72°C,  
20 followed by a final extension for 4 minutes at 72°C. Typing of HPV was performed by using PCR type-specific primers against HPV 16, 18, 31, and 33 (6/11, 35, 45, 51, 52, 58), as described above.

#### 25 Results

Originally 190 patients were biopsied after being given the diagnosis CIN I, CINII, or CIN III by cytology. A high-grade lesion was confirmed by histologically examination, 150 samples diagnosed as  
30 CIN III (78.9%). Biopsy 2, taken before conization, was used for RNA analysis. However, histological examination of this biopsy diagnosed only 53 samples of the originally 150 as CIN III [54 were given no diagnosis, 24 diagnosed as CIN II, 18 as CIN I, and 4  
35 as HPV/condylom]. The number of CIN II samples increased from 16 (8,4%) to 30 (15,8%) [by Histology I

- 87 -

Type-specific PCR detected 85 cases containing HPV; 66 having HPV 16, 10 HPV 18, 14 HPV 31, 7 HPV 33. 12 cases had multiple infection: 3 with HPV 16+18; 4 with HPV 16+33, 5 with HPV 16+31. 20 no diagnosis.

5

Example 4

HPV detected by PreTect HPV-Proofer and PCR compared to cytology and histology:

10 Normal and ASCUS samples (including borderline smears) were determined by cytology. All samples were tested with consensus PCR and PreTect HPV-Proofer but only the consensus positive samples were typed by PCR. The CIN 3 and cancer samples were determined by  
15 histology and all the samples were tested with all three methods. The results are shown in Figure 6. Concordance between real-time multiplex NASBA and PCR compared to cytology or histology is shown in Table 15 below.

20

Table 15: Concordance between real-time multiplex NASBA and PCR compared to cytology or histology

Cytology/Histology	Concordance <sup>a</sup> (Number)	Concordance <sup>b</sup> (Number)
Normal	98.2% (4043)	42.8% (138)
ASCUS <sup>c</sup>	94.5% (55)	78.6% (14)
CIN 3	94.3% (53)	93.2% (44)
Cancer	99.0% (196)	98.8% (170)

25

30 Only samples positive by Gp5+/6+ PCR have been typed.  
<sup>a</sup> Including PCR and real-time multiplex NASBA positive and negative samples. <sup>b</sup> Including only PCR and / or real-time multiplex NASBA positive samples. <sup>c</sup> ASCUS excluding borderline smears.

35

Example 5

The invention provides a kit for detection of mRNA transcripts from the E6 gene(s) of HPV the kit comprising one or more of, two or more of and preferably all of the following primer pairs and accompanying identification probes.

HPV 16:HPV16.txt 7905 b.p

10

HPV16P2:

p2:116 (20) GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAGAAA

16 p1 (no7)

15 AATTCTAATACGACTCACTATAGGGAGAAGG ATT CCC ATC TCT ATA TAC TA (51  
baser)

HPV16PO2:

po:230 (20) TATGACTTTGCTTTTCGGGA

H16e6702po

20

HPV 18:HPV18.txt 7857 b.p

HPV18P2:

25 p2:698 (22) GATGCAAGGTCGCATATGAGGAAAACGATGAAATAGATGGAG

H18e6702p2

HPV18P4:

p1:817 (20)

30 AATTCTAATACGACTCACTATAGGGAGAAGGGGTCGTCTGCTGAGCTTTCT

H18e6703p1 (Multiplex)

HPV18PO2:

35 po:752 (21) GAACCACAACGTCACACAATG

H18e6702po

HPV 31:HPV31.txt 7912 b.p

40

HPV31P3:

p2:617 (20) GATGCAAGGTCGCATATGAGACTGACCTCCACTGTTATGA

- 89 -

H31e6703p2  
 p1:766 (20)  
 AATTCTAATACGACTCACTATAGGGAGAAGGTATCTACTTGTGTGCTCTGT  
 H31e6703p1  
 5  
 HPV31PO4:  
 po:686 (26) GGACAAGCAGAACCGGACACATCCAA  
 H31e6704po  
 10  
 HPV 33:HPV33.txt 7909 b.p  
 HPV33P1:  
 p2:618 (22) GATGCAAGGTCGCATATGAGTATCCTGAACCAACTGACCTAT  
 15 H33e6701p2  
 p1:763 (19)  
 AATTCTAATACGACTCACTATAGGGAGAAGGTTGACACATAAACGAACTG  
 H33e6701p1  
 20 HPV33PO3:  
 po:699 (23) GGACAAGCACAACCAGCCACAGC  
 H33e6703po

25 As alternative to the probes shown above the kit may  
 optionally include one or more of the following  
 molecular beacon probes:

#### Molecular Beacon Probes:

30 H16e6702mb2-FAM ccagctTATGACTTTTGCTTTTCGGGAagctgg  
 H18e6702mb1-TxR cgcattGAACCACAACGTCACACAATGcatgag  
 35 H31e6704mb2-FAM ccgtcgGGACAAGCAGAACCGGACACATCCAAcgacgg  
 H33e6703mb1-FAM ccaagcGGACAAGCACAACCAGCCACAGCgcttgg

40 Preferably the kit of the invention also includes the  
 following primer pair and probe. :

- 90 -

HPV45: HPV45.txt 7858 bp (X74479)

HPV45P1:

p2:430 (21): GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCAGAAA

5 H45e670lp2

p1:527 (22):

AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTCTTGCCGTGCCTGGTCA

H45e670lp1

10 HPV45PO1:

po:500 (20): GTACCGAGGGCAGTGTAATA

H45e670lpo

15 The HPV 45 probe above may be replaced by an HPV  
molecular beacon probe as follows:

H45e670lmb1 cgatcgGTACCGAGGGCAGTGTAATAcgatcg

20 In addition the kit may include one or more of the  
following primer pairs and accompanying identification  
probes depending on the geographical area of use of the  
kit.

HPV52: HPV52.txt 7942 bp (X74481)

25

HPV52P1:

p2:144 (22): GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTGGAAGAAT

H52e670lp2

p1:358 (18): AATTCTAATACGACTCACTATAGGGAGAAGGCCCTCTCTTCTAATGTTT

30 H52e670lp1

HPV52PO1:

Po:296 (20): GTGCCTACGCTTTTTATCTA

H52e670lpo

35

HPV58 HPV58.txt 7824 bp (D90400)



- 91 -

HPV58P2:  
p2:173 (18): GATGCAAGGTCGCATATGAGTCTGTGCATGAAATCGAA  
H58e6702p2  
p1:291 (18):  
5 AATTCTAATACGACTCACTATAGGGAGAAGGAGCACACTTTACATACTG  
H58e6702p1

HPV58PO2:  
po:218 (22): TTGCAGCGATCTGAGGTATATG  
10 H58e6702po

HPV51 HPV51.txt 7808 bp (M62877)

HPV51PA/P:  
15 p2:655 (23): GATGCAAGGTCGCATATGAG AGA GGA GGA GGA TGA AGT AGA TA  
H51e6702p2  
p1:807 (20): AATTCTAATACGACTCACTATAGGGAGAAGG GCCCATTAACAT CTG  
CTG TA H51e6701p1

20 HPV51POA:  
po:771 (24): TGG CAG TGG AAA GCA GTG GAG ACA  
H51e6702po

25 The probes shown above may be replaced in the kit by the  
following molecular beacon probes:

H52e6701mb1 cgatcgGTGCCTACGCTTTTTATCTAcgatcg

H58e6702mb1 ccgtcgTTGCAGCGATCTGAGGTATATGcgacgg

30 H51e6702mb1 cgatcgTGG CAG TGG AAA GCA GTG GAG ACAcgatcg

35

**CLAIMS**

1. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or E6 mRNA are scored as being at risk of developing cervical carcinoma.

2. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of human papillomavirus (HPV) and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA;

Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

- 93 -

3. A method according to claim 1 which further comprises screening for expression of p16<sup>ink4a</sup>, wherein subjects positive for expression of L1 and/or E6 mRNA and positive for expression of p16<sup>ink4a</sup> are scored as  
5 being at risk of developing cervical carcinoma.

4. An *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises  
10 screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV or a  
15 modified episomal HPV genome.

5. An *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises  
20 screening the subject for expression of mRNA transcripts from the E6 gene of human papillomavirus, wherein subjects positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome.

25  
6. An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma, which method comprises screening the subject for expression of mRNA transcripts of the E6  
30 gene of HPV and sorting the subject into one of two categories of risk for development of cervical carcinoma based on expression of E6 mRNA, wherein individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified  
35 episomal HPV genome and are therefore classified as high risk for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA

- 94 -

are scored as not carrying integrated HPV or a modified episomal HPV genome and are therefore classified as no detectable risk for development of cervical carcinoma.

5

7. An *in vitro* method of identifying human subjects having abnormal cell changes in the cervix, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV, wherein individuals positive for expression of E6 mRNA are identified as having abnormal cell changes in the cervix.

8. A method according to any one of claims 5 to 7 wherein the human subjects are subjects previously identified as infected with human papillomavirus DNA in cells of the cervix.

9. A method according to any one of claims 5 to 7 wherein the human subjects are subjects having a previous diagnosis ASCUS, CIN 1 lesions or condyloma.

10. A method according to any one of claims 4 to 9 wherein individuals positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33 or 45 are scored as carrying integrated HPV.

11. A method according to any one of claims 1 to 4 which comprises screening for L1 mRNA expression using a technique which is able to detect L1 mRNA from substantially all known HPV types.

12. A method according to any one of claims 1 to 11 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from at least one cancer-associated HPV type,

- 95 -

13. A method according to claim 12 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31, 33, and preferably 45.

5

14. A method according to any one of claims 1 to 13 wherein screening for L1 and/or E6 mRNA expression is carried using an amplification reaction to amplify of a region of the mRNA, together with real-time detection of the products of the amplification reaction.

10

15. A method according to claim 14 wherein screening for L1 and/or E6 mRNA expression is carried using real-time NASBA.

15

16. A kit for use in the detection of mRNA transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31, 33, and preferably 45 and one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31, 33, and preferably 45.

20

25

1/6

FIG. 1A.

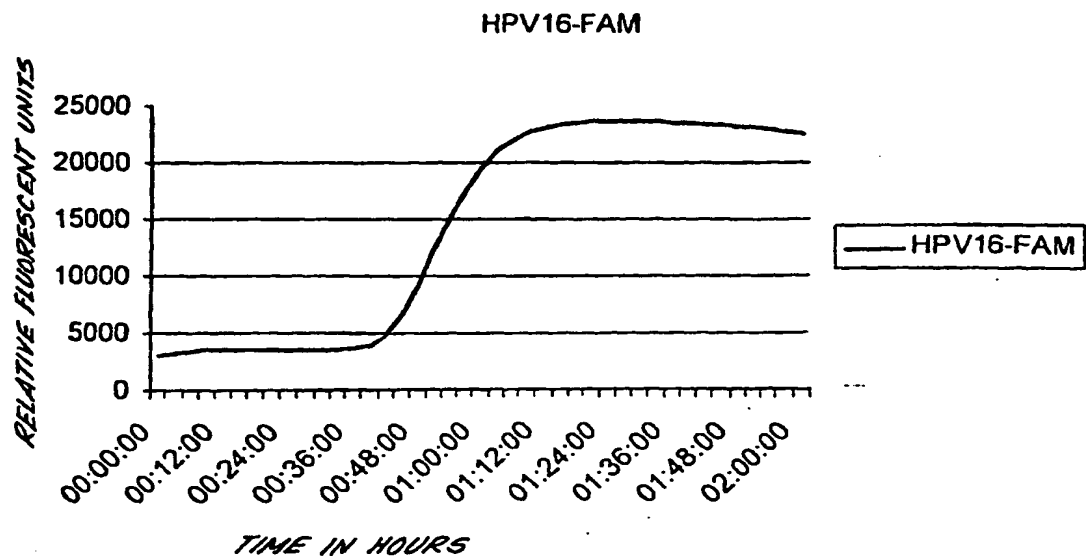
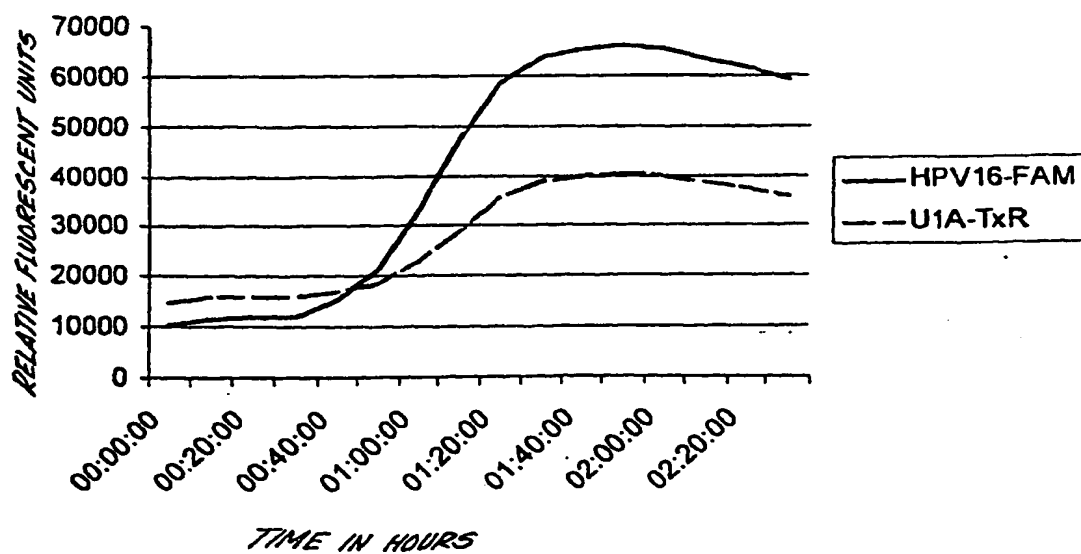


FIG. 1B.



2/6

FIG. 2A.

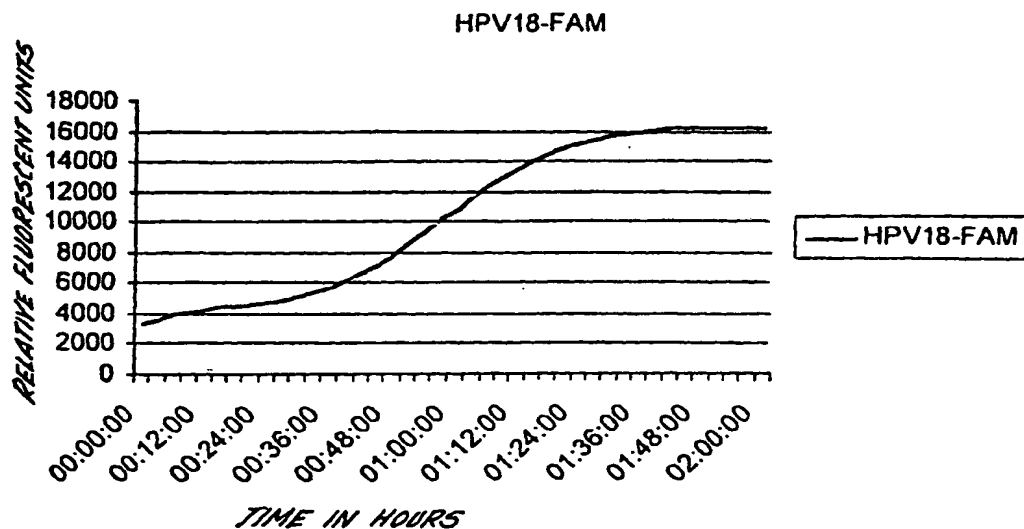
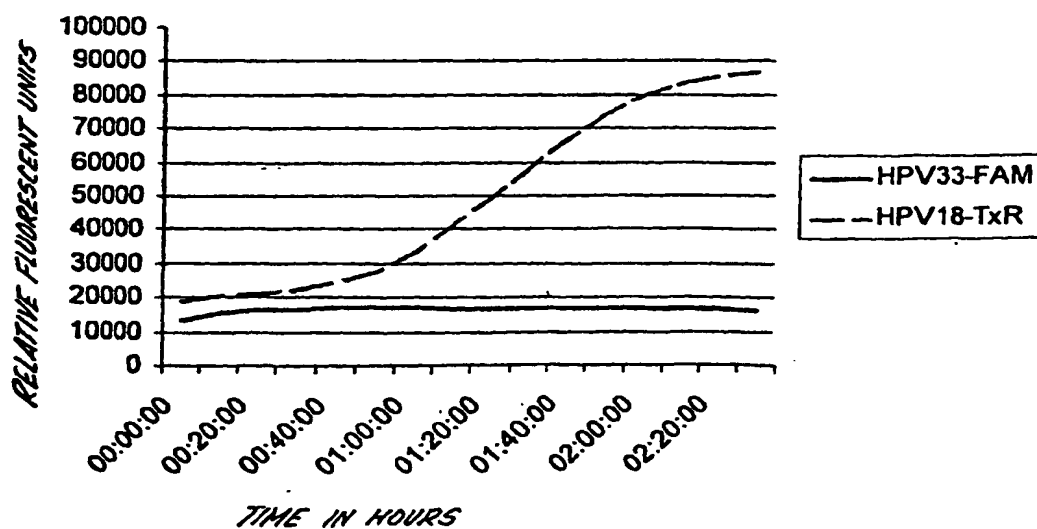


FIG. 2B.



3/6

FIG. 3A.

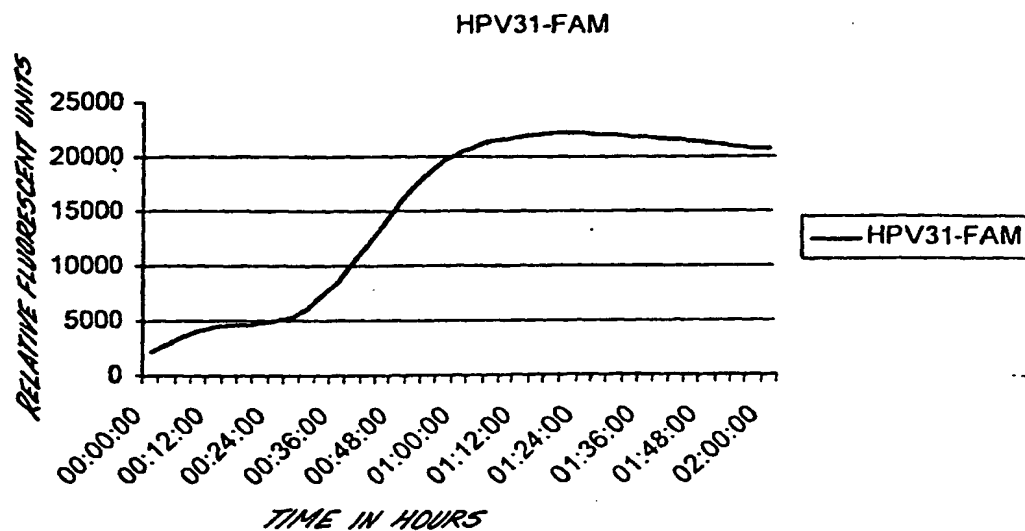
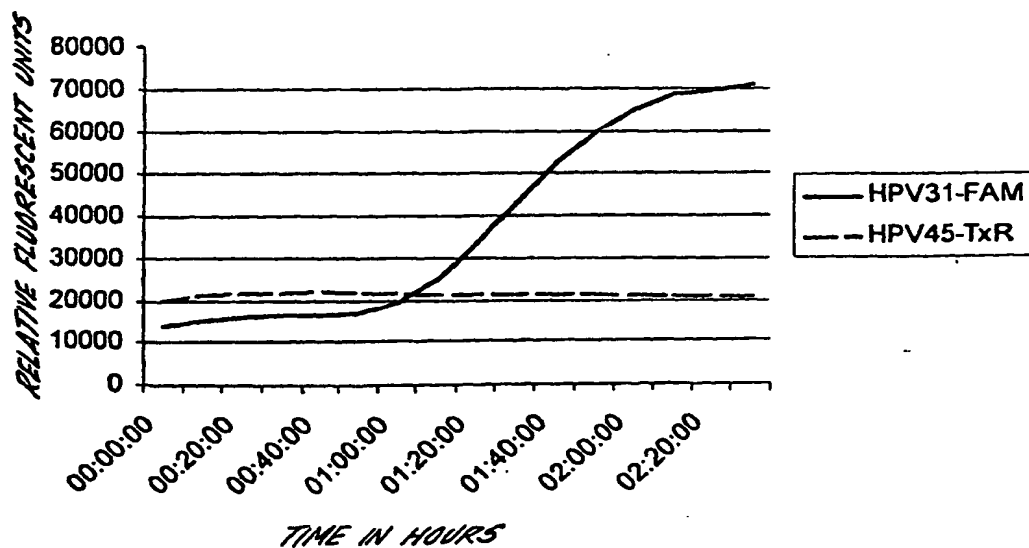


FIG. 3B.





4/6

FIG. 4A.

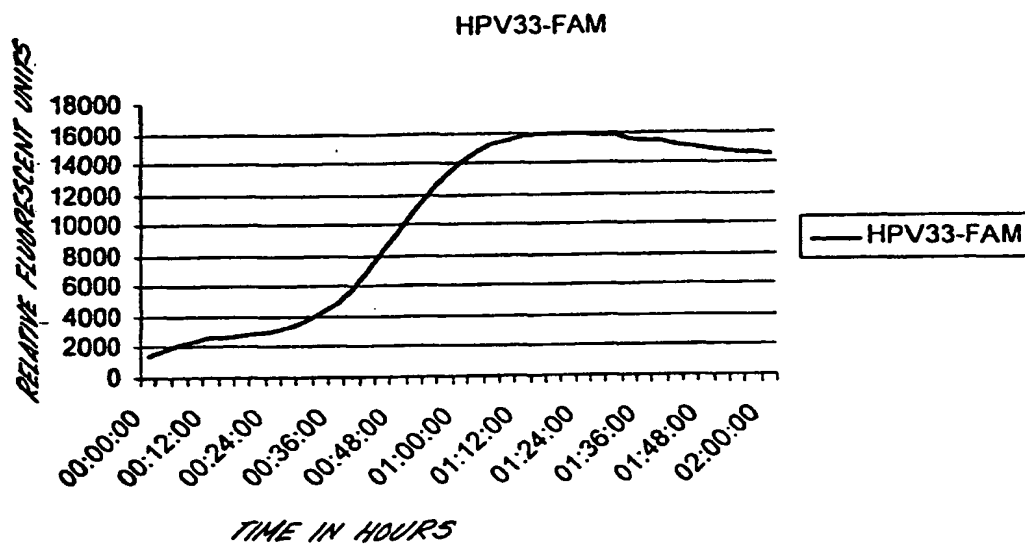
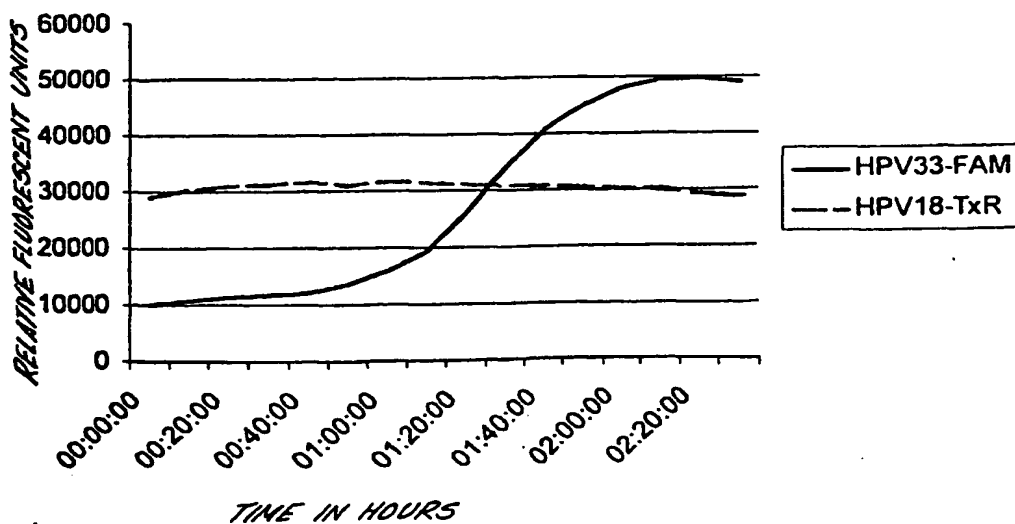


FIG. 4B.



5/6

FIG. 5A.

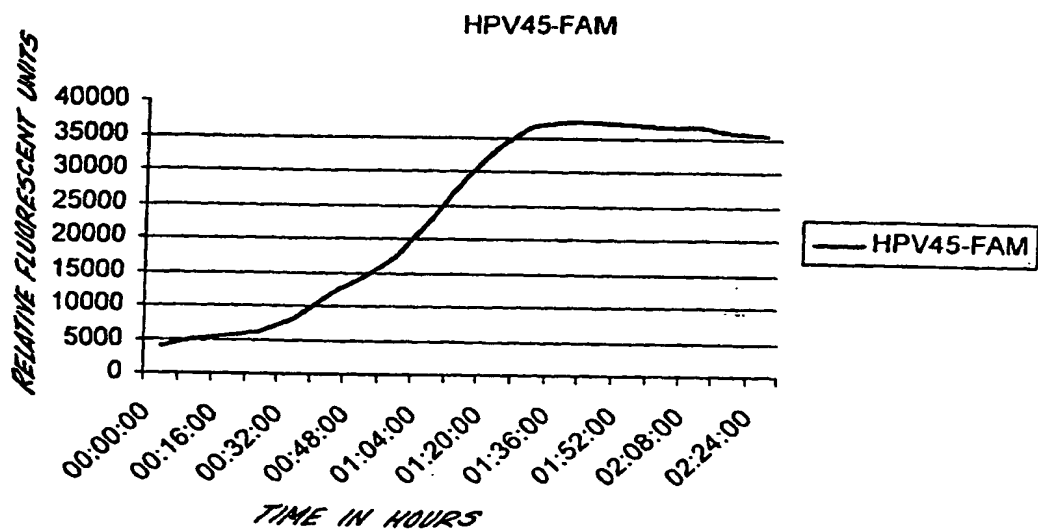
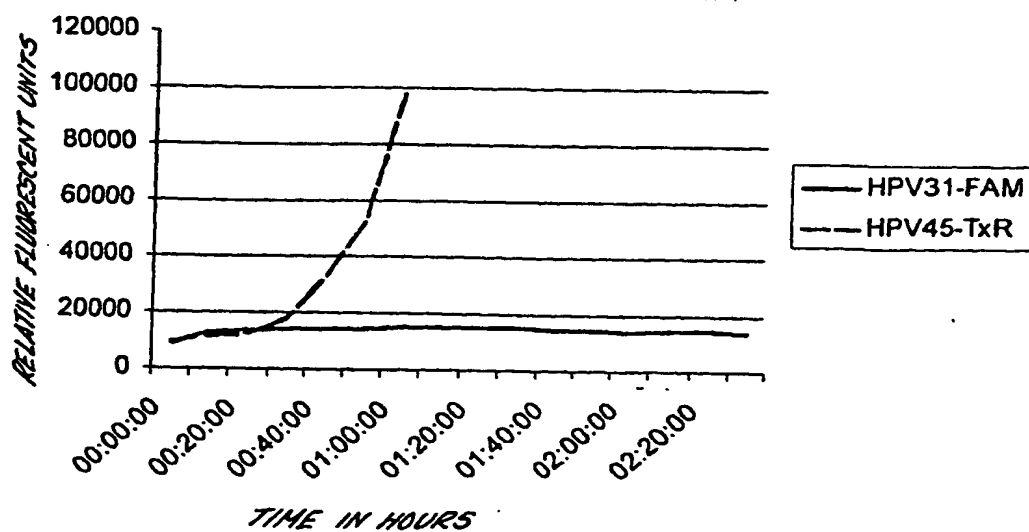


FIG. 5B.



6/6

FIG. 6.

